PLASMID INSTABILITY: MEASUREMENT AND USE IN ANTIMICROBIAL ACTION

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DECLARATION AND CERTIFICATE

I hereby certify that the work embodied in this thesis is the result of my own investigations except where information obtained from colleagues has been acknowledged. This work has not already been accepted in substance for any degree and is not currently submitted in candidature for any other degree.

Nicola Crewe

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ABSTRACT

The discovery of antibiotics in the early 20th century revolutionised medicine, but quickly bacteria began to demonstrate resistance to these agents. Antibiotic resistance is still on the increase, and soon, if this trend continues, bacterial infections may not be treatable with antibiotics. In an effort to prevent this occurring, the search for novel antibiotics is under way and new antibiotic targets are being considered. One target that has not been studied in depth is the partitioning systems of bacterial plasmids. Disruption of plasmid partition would prevent effective plasmid inheritance, which, in the case of resistance plasmids, would render the host cell antibiotic sensitive.

The aim of this study was to determine whether plasmid partitioning is a viable target for a new antibiotic. A series of plasmids that contained genetically altered partitioning systems was used, which provided a range of plasmid stabilities. The plasmids all conferred antibiotic resistance on the host cell, allowing the effect of plasmid instability on a population grown in the presence of antibiotics to be determined.

Several different methods of cell culture were used. Simple batch culture experiments allowed the observation that few plasmid-free cells were produced from cells containing plasmids that had a functional system. In contrast, plasmids containing a faulty system were found to be rapidly lost from the host cells. Steady-state chemostat culture was used to provide a simple model of a clinical infection. The formation of equilibria between plasmid-free and plasmid-bearing cells was observed, and the cultures contained a large proportion of plasmid-free cells when the experiments involved unstable plasmids. The slow growth rate of cultures in the chemostat was seen to dramatically affect the inheritance of plasmids relying on random distribution.

Finally, cultures were subjected to washout in order to determine their maximum specific growth rate (μ_{max}). While the results from these experiments are not entirely conclusive, there is a strong indication that the growth rate of cultures containing unstable plasmids grown in the presence of antibiotics is reduced.

The Unknown

As we know,
There are known knowns.
These are things we know we know.
We also know
There are known unknowns.
That is to say
We know there are some things
We do not know.
But there are also unknown unknowns,
The ones we don't know
We don't know.

Donald H. Rumsfeld

CONTENTS

CHAPTER ONE – GENERAL INTRODUCTION TO ANTIBIOTIC RESISTANCE AND PLASMID STABILITY MECHANISMS

1.1 The discovery of antibiotics

Much of modern medicine is dependent on the action of chemotherapeutic agents, chemicals that can kill or inactivate microorganisms without causing undesirable side effects or damage to the host. The German physician Paul Ehrlich was the first to consider the possibility of selective chemicals; he envisaged "magic bullets" that could affect bacteria but not humans. By 1904 he had discovered that the dye Tryptan Red was active against the trypanosome that causes African sleeping sickness, and by 1910 the compound arsphenamine was available for the treatment of syphilis as a direct result of Ehrlich's work (Stratton, 1996).

It did not take much longer for more chemotherapeutic agents to be discovered. In 1929 Alexander Fleming discovered penicillin, although it took a further thirteen years before the first patient was treated with this antibiotic, and then only after additional work had been carried out by Howard Florey and Ernst Chain (Radetsky, 1996). In Germany, Gerhard Domagk discovered that prontosil rubrum was able to cure streptococcal infections in mice (Stratton, 1996). By 1953 streptomycin, chloramphenicol, neomycin, terramycin and tetracycline had been discovered and the era of antibiotics was truly under way (Neu, 1994).

1.2 The action of ampicillin and chloramphenicol on the bacterial cell

The modes of action of antibiotics are as diverse as the compounds themselves. In all cases they act against a structure within the bacterial cell that has no counterpart within eukaryotic cells. The antibiotics that cause the fewest side effects are those that act against the bacterial cell wall, a structure not seen in any form within eukaryotic cells (Stratton, 1996). The ß-lactams are the largest group of antibiotics that function in this manner, and the mode of action of ampicillin, a member of the ß-lactam group, will be described later. Another successful group of antibiotics are those that target the bacterial ribosome. Although ribosomes are present within eukaryotic cells, their structure is very different from the prokaryotic ribosome, hence the ability of antibiotics

to distinguish between the two types. Chloramphenicol is an example of an antibiotic that functions against the prokaryotic ribosome (Pongs *et al*., 1973).

The ß-lactam group is made up of two classical families. The penicillin family includes the first ß-lactam antibiotic, benzylpenicillin, and ampicillin (Fig. 1.1); while the cephalosporin family includes carbacephem and cephalothin. Various non-classical ß-lactams have also been developed, one example of which is monobactam (Livermore & Williams, 1996).

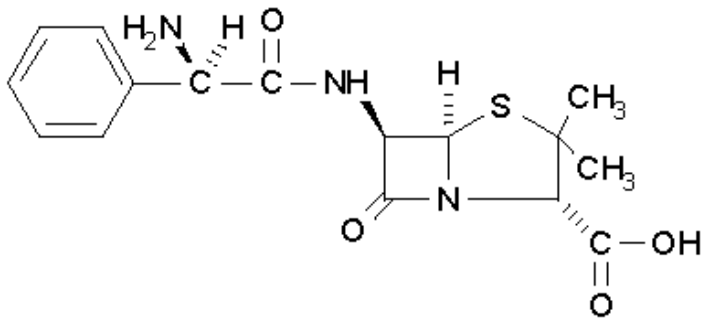


Fig. 1.1: The structure of ampicillin

Because of the vast number of compounds in the ß-lactam group, this class of antibiotics are used to treat approximately 50% of all systemic infections. The universal feature of the group is the ß-lactam ring, which is the structure responsible for the antimicrobial action. The ß-lactam ring binds to, and acylates, enzymes known collectively as penicillin-binding proteins. These are involved with the production of peptidoglycan, the major polymer of the bacterial cell wall. The cell wall cannot form correctly without the action of these enzymes, and so the cell wall is weakened and the cell cannot survive against osmotic forces. ß-lactam antibiotics are described as bactericidal because of their ability to clear bacterial infections with no assistance from the host immune system (Livermore and Williams, 1996).

Other antibiotics are described as bacteriostatic because they prevent the cell from replicating while the antibiotic is present in their growth environment, but once the antibiotic is removed the bacteria can start replicating again. These antibiotics will therefore not remove a bacterial infection on their own, but require assistance from the

host immune system or co-administration with a bactericidal antibiotic (Eliopoulos & Moellering, 1996). One example of a bacteriostatic antibiotic is chloramphenicol (Fig. 1.2), which reversibly binds to the 50S subunit of the bacterial ribosome to prevent the formation of a peptide bond during protein synthesis (Pongs *et al*., 1973).

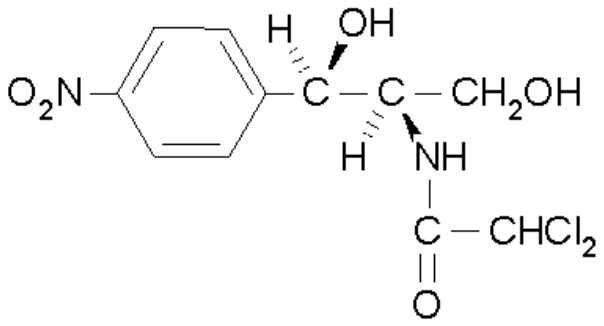


Fig. 1.2: The structure of chloramphenicol

Chloramphenicol also affects other processes in bacterial cells, for example the bacterial translocase reaction (Stratton, 1996), and the combination of effects means that long-term exposure to chloramphenicol can lead to cell death. However, when used to treat clinical infections, the half-life of chloramphenicol in blood serum has been calculated as a maximum of 4 hours (Gerding *et al*., 1996), and this exposure time is not long enough to eradicate the infection. Successful treatment of infections with chloramphenicol, or any other bacteriostatic antibiotic, therefore relies on additional assistance from either the host immune system or other antibiotics (Eliopoulos & Moellering, 1996).

1.3 The emergence of antibiotic resistance

Prior to the discovery of antibiotics, serious bacterial infections had to be treated with major surgery such as amputation. Infections caused by *Staphylococcus aureus* were fatal in 80% of cases, and *Streptococcus pyogenes* was implicated in over 50% of all post-birth deaths. However, as a direct result of the discovery of antibiotics, the number of deaths caused by bacterial infections dropped dramatically (Wilson, 2002). There was such conviction that antibiotics would permanently remove the threat of bacterial

infections, that in 1969 the then US Surgeon General William Stewart stated, “it is time to close the book on infectious diseases” (Meyer, 2001).

However, the book was not destined to remain closed. The first case of successful treatment of Gonorrhoea with sulphonamides was in 1936, but only six years later almost all strains of *Neisseria gonorrhoeae* were found to be resistant to this group of antibiotics (Amsterdam, 1996). This emergence of resistance did not appear to cause overt concern, mainly due to the large number of novel antibiotics being discovered every year. But by the end of the 1960’s, sources of new antibiotics were running dry and antibiotic resistance was still on the increase (Wilson, 2002).

The emergence of antibiotic resistance came as a surprise for many, especially since there was worldwide insistence that the threat of bacterial infections had been permanently removed. The consideration of antibiotics as ‘wonder drugs’ led to their indiscriminate use (Stratton, 1996), even though some had voiced their caution against this attitude. Indeed, Alexander Fleming himself foresaw the emergence of antibiotic resistance and in 1945 stated; “The greatest possibility of evil in self-medication is the use of too-small doses, so that, instead of clearing up infection, the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed on to other individuals and perhaps from there to others until they reach someone who gets a septicaemia or pneumonia which penicillin cannot save” (Anon, 1945).

1.4 The acquisition and transfer of antibiotic resistance

The fast evolution rates of bacteria provide a huge opportunity for natural selection to take its course and, as a result, organisms that were capable of withstanding antibiotics quickly arose (Rice & Bonomo, 1996). In addition, antibiotic resistance had always existed, and the excessive use of antibiotics increased the spread of this resistance. The different routes of acquisition of antibiotic resistance are discussed below.

Rapid evolution through mutation of the bacterial chromosome has been observed for many different bacterial traits, not just in the case of antibiotic resistance (Hogan & Kolter, 2002). While most mutations produce little useful alteration within the

organism, occasionally mutations can alter binding sites, proteins or other structures within the bacterial cell. A mutation within the structure to which the antibiotic usually binds will prevent correct binding leading to resistance. This has been observed in *Helicobacter pylori*, where a mutation in the 16S rRNA gene prevents the binding of tetracycline (Trieber & Taylor, 2002). Mutation can also lead to overproduction of proteins, and if this protein is the antibiotic target then excess proteins may prevent the organism from being overcome by the antibiotic (Hogan & Kolter, 2002). *Escherichia coli* 1810 is resistant to trimethoprim because the target enzyme of the antibiotic, dihydrofolate reductase, is overproduced (Flensburg & Skold, 1984).

Many organisms show intrinsic resistance to antibiotics; in other words, the organism has always been resistant to the drug because of its genetic structure. One source of intrinsic resistance is from cells that naturally produce antibiotics as a defence mechanism. For example, *Streptomyces griseus*, the organism from which the antibiotic streptomycin was first isolated, contains resistance mechanisms in order to prevent cell death from the antibiotic it produces (Sugiyama & Nimi, 1987). Another type of intrinsic resistance is found in *Stenotrophomonas maltophilia*, where intrinsic efflux mechanisms and production of ß-lactamases are part of the plethora of mechanisms that make this organism one of the most dangerous nosocomial pathogens (Zhang *et al*., 2001).

Once the genes responsible for conferring antibiotic resistance on a host cell are present in the bacterial population, the transfer of these genes to other cells within the population can occur extremely rapidly. Replication and division will ensure that antibiotic resistance genes are transferred to daughter cells, while the transfer of plasmids or transposons between unrelated cells will further increase the spread (Hogan & Kolter, 2002). This horizontal transfer can even allow genes to spread within different species of bacteria. Transposons are frequently found to contain antibiotic resistance genes, and these genetic elements are able to excise themselves and move from one section of a chromosome to another. This also allows for transfer during bacterial conjugation, where parts of the bacterial chromosome are transferred from one cell to another. If a transposon should incorporate itself into a bacterial plasmid, then transfer of genetic material can occur through transduction and transformation as well as conjugation. It must also be remembered that transposons can excise themselves

from a plasmid and move back into the chromosome (Hayes, 2003), and therefore a cell containing a newly-acquired plasmid can soon also bear chromosomally-encoded antibiotic resistance.

Another recently discovered source of antibiotic resistance is unrelated to the transfer of antibiotic resistance genes, but instead appears to be a natural capability of certain organisms. There is a possibility that physiological changes within the cell can alter the receptiveness of the organism to antibiotics. A decrease in antibiotic sensitivity has been seen in biofilm bacteria such as *Staphylococcus epidermidis*, probably as a result of the slower growth rates, decreased diffusion of antibiotics and the accumulation of resistance enzymes in biofilm cultures (König *et al*., 2001; Hogan & Kolter, 2002).

1.5 Resistance to ampicillin and chloramphenicol

Resistance to ampicillin and chloramphenicol is widespread because these are two of the earliest antibiotics discovered, and therefore have been in use for a substantial period of time (Stratton, 1996).

Resistance to ampicillin is caused by production of the ß-lactamase enzyme. These cleave the ß-lactam ring of the antibiotic, preventing the compound from binding to the penicillin-binding proteins. There are several groups of ß-lactamases defined by the ß-lactams against which they are most active. The class A TEM-1 enzyme was originally found in a single isolate of *E. coli* in 1965. It is a plasmid-encoded enzyme commonly found in Gram-negative rods; indeed 50 to 60% of ampicillin-resistant *E. coli* isolates worldwide contain a plasmid that carries the genetic coding for this ß-lactamase (Livermore and Williams, 1996).

Resistance to chloramphenicol is caused by the acquisition of the genes coding for chloramphenicol acetyl transferase (CAT). CAT provides resistance to chloramphenicol by enzymatic inactivation; it acetylates chloramphenicol thus preventing its binding to the ribosome. Study of clinical isolates bearing chloramphenicol resistance have shown that there are seven classes of *cat* genes, which can be plasmid or chromosomally mediated. However, all classes of enzymes show almost exactly the same function (Rice and Bonomo, 1996).

1.6 The quest to isolate novel antibiotics

With the dramatic increase in antibiotic resistance, the challenge is now on to find new types of chemotherapeutic agents. In the 1960's the focus moved towards altering existing antibiotics to create new drugs with basically the same action but a different structure in order to circumvent the resistance mechanisms (Livermore & Williams, 1996). An example of chemical alteration of existing antibiotics is the production of atypical tetracyclines from chlortetracycline. Although all the compounds within the tetracycline group have essentially the same structure, the atypical tetracyclines attack the cytoplasmic membrane rather than the ribosome, and can therefore avoid recognition by resistance mechanisms (Stratton, 1996). This strategy worked for a short period of time before, predictably, resistance began to appear against these new compounds (Livermore & Williams, 1996).

Another focus for drug development occurred with the discovery of compounds that inhibit the mechanisms responsible for antibiotic resistance. These compounds can allow obsolete antibiotics, such as penicillin, to be used again. Several compounds of this type have been discovered, the most notable being the ß-lactamase inhibitors, but again resistance soon spread (Livermore & Williams, 1996). We are now reaching a situation where some infections cannot be easily treated and are once again becoming life threatening (Wilson, 2002). In fact the Joint Expert Technical Advisory Committee on Antibiotic Resistance has stated, "resistance has emerged for all known antibiotics in use" (JETACAR, 1999).

One small ray of light emerged in the fight against antibiotic resistance a few years ago. A new class of antibiotics known as the oxazolidinones was first described in 1987, although they were considered too harmful for human use (Stratton, 1996). However, in April 2000, one of the antibiotics from this class, known as Zyvox™, received FDA approval and is now available for use. It is commonly described as a breakthrough due to its being "the first antibiotic in a completely new class in 35 years" (Pharmacia, 2003).

1.7 The potential of plasmids as a target for a novel antibiotic

While the discovery of the oxazolidinone class of antibiotics brings some hope to the current situation, the search for new antibiotics must continue to ensure that we will not return to a pre-antibiotic era.

Antibiotic screening techniques have been modernised, and now focus on the use of target-based searches as opposed to the random screening of chemicals (Wilson, 2002). However, for this new approach to work, potential targets for antibacterial action must first be determined. The most essential feature of a target is that it must not have an analogue in eukaryotic cells. In this way, the microorganism is selectively killed while the host remains unharmed (Rosamond & Allsop, 2000).

Many of the obvious targets for antibiotics have been exhaustively researched and exploited, for example, the bacterial cell wall is the target for the both the ß-lactam and cephalosporin groups of antibiotics (Livermore & Williams, 1996; Stratton, 1996). However, there are other targets, such as the bacterial plasmid, that have not yet been fully considered.

Plasmids can be found in single celled eukaryotic organisms such as *Saccharomyces cerevisiae*, but are not found in multicellular eukaryotic organisms. They commonly carry genes that confer antibiotic resistance (section 1.4), and sometimes provide the host cell with increased pathogenicity and enhanced growth (Table 1.1). In general, plasmids also contain systems that ensure efficient replication and transfer of the plasmid to daughter cells during cell division (section 1.9).

Organism	Plasmid name	Function	Reference
Chlamydia trachomatis trachoma biovar	pCT	Cryptic, but thought to be involved with survival in the environment, and causes changes in cell physiology during infection	Comanducci *et al*., 1993
Shigella flexneri	pWR100	Allows entrance into epithelial cells, and induces apoptosis in macrophages.	Bruchrieser *et al*., 2000
Salmonella typhimurium	pSLT	For the infective process after penetration of the gut wall	Cerin & Hackett, 1993
Bacillus anthracis	pXO1	Toxin production	Fouet *et al*., 1994
Clostridium perfringens	cpe	Enterotoxin production	Brynestad *et al*., 2001

Table 1.1: To show a range of functionality exhibited by bacterial plasmids that provide a benefit to the host organism

If the inheritance and transfer of plasmids can be disrupted, then plasmid-free cells will be produced. The loss of plasmids such as those described in Table 1.1 will render cells non-pathogenic, while the loss of an antibiotic-resistance conferring plasmid will render the cell susceptible to antibiotics.

It has also been suggested that the systems that ensure plasmid inheritance and chromosome partition during cell division are very closely related (Mercado-Blanco & Olivares, 1994). For example, the MukB protein of *E. coli* appears to be responsible for moving the chromosome copies to the plane of cell division (Hiraga, 1992), and *spoOJ* and *soj* are responsible for translocating the chromosome copies into *Bacillus subtilis* prespores (Sharpe & Errington, 1996). Both of these systems appear to function in a similar way to that of active plasmid partitioning systems (section 1.9.4). Site-specific recombination (section 1.9.1) is also thought to be involved in chromosomal partitioning, where the *dif* site of *E. coli* is seen to function as a specific recombination site (Wake & Errington, 1995). Therefore a compound that disrupts plasmid inheritance

may also affect chromosome partitioning, and as a result could be useful as a general antibacterial.

Hence, the genes that regulate the transfer of plasmids to daughter cells become a viable target, not for a true antibiotic, but for a compound to be administered in conjunction with a true antibiotic.

1.8 The aims and objectives of this study

The main aim of this study was to determine whether reducing the efficiency of plasmid inheritance would produce a measurable increase in the number of antibiotic-sensitive cells within a bacterial culture.

If an increase in antibiotic sensitivity was observed, then the second aim was to show that chemicals known to have an effect on plasmid replication, such as acridine orange or bromidoprim (Marchese *et al*., 1996), could affect plasmid inheritance. This would suggest that plasmid stability systems could be affected by external influences. If this were the case, then the final aim was to produce a method to screen for potential destabilising compounds.

In order to fulfil the main aim of the study, the first objective was to obtain plasmids that showed a range of stabilities. A study carried out by Williams *et al*. (1998) utilised a series of genetically altered plasmids that showed different levels of plasmid stability. All of these plasmids encoded resistance to ampicillin, and as a result a decrease in plasmid stability would manifest itself as an increase in ampicillin sensitivity.

As these plasmids showed resistance to a single antibiotic, a study based on these plasmids alone would be limited when trying to apply the results to a general discussion on the use of destabilising plasmids as a method of restoring any type of antibiotic sensitivity. Therefore, the second objective of this section of work was to engineer the genetically altered plasmids to confer resistance to another antibiotic. The antibiotic chosen was chloramphenicol, as this has a very different mechanism of action to ampicillin and is a bacteriostatic rather than bactericidal antibiotic.

Once both sets of plasmids were available, the next objective was to carry out a preliminary determination of the effects of plasmid instability on antibiotic resistance. This was carried out by growing plasmid-containing bacteria in batch culture conditions. Batch culture is a method that benefits from a short experimental time, therefore allowing preliminary data to be obtained rapidly.

The fourth objective was to try to observe the effects of plasmid instability on antibiotic sensitivity in a 'natural' situation. If the destabilisation of plasmids were shown to decrease antibiotic resistance, then the most obvious application would be to the treatment of resistant bacterial infections. Batch culture, while a fast experiment, does not accurately portray the growth of bacteria during an infection, while steady-state chemostat culture provides a more realistic growth environment, with limited nutrient availability, a regulated pH and removal of waste products. Observation of the growth of bacteria containing a destabilised plasmid under steady-state chemostat culture conditions would provide an indication as to whether plasmid destabilisation would be a useful target for an antimicrobial agent.

While steady-state chemostat culture would provide an indication of the effect of destabilisation on antibiotic resistance; it does not supply a numerical value as to the extent of the effect. Washout cultures can be used to calculate the growth rate of a bacterial population, which is a numerical value that can be used for direct comparison between cultures. Therefore, the fifth objective was to determine the growth rates of all the plasmid-containing cultures in the presence and absence of antibiotics. A noticeable difference in growth rates between cultures containing stable or destabilised plasmids would indicate that plasmid destabilisation is having an effect on the growth of the cultures.

Fulfilling the objectives described above would complete the first aim of the project, and as a result it would be possible to comment on whether plasmid destabilisation is capable of affecting antibiotic resistance within a bacterial culture.

The second and third aims were not addressed during the course of this study, as fulfilment of the main aim took longer than was anticipated. However, the work required to fulfil the second and third aims was considered in detail, and can be found in

chapter eight. In order to fully appreciate how a destabilising agent could affect plasmid inheritance, it is necessary to understand the ways in which plasmid stability is achieved. There are many different mechanisms by which this occurs; an overview is given in Table 1.2, while the mechanisms are described in detail in section 1.9.

1.9 Plasmids and their stability mechanisms

Plasmids are extra-chromosomal genetic elements of DNA or RNA that are capable of replicating independently from the host chromosome (Lederberg, 1998), but which usually replicate in tandem with the chromosome to ensure correct inheritance during cell division (Paulsson & Ehrenberg, 1998). They are frequently circular molecules, although some exist as a linear strand (Lederberg, 1998). Their size can vary enormously from a few hundred base pairs to a molecule almost as large as the chromosome itself, and they can be present in the dividing cell as a single copy, or up to 200 copies as in the case of pUC18 (Summers, 1991).

For a plasmid to function correctly, an origin of replication and a negative-feedback control system to limit the plasmid copy number are all that is required. A plasmid containing only these systems is known as a basic replicon (Nordström & Austin, 1989; Thomas & Jagura-Burdzy, 1992). However, a plasmid normally contains more systems than those seen within the basic replicon; they also frequently contains systems that allow for more efficient replication and partition, and sometimes genes that provide other benefits are present (section 1.7).

The presence of a plasmid creates a metabolic burden for the host cell, due to the energy required for replication and protein production (Cooper *et al*., 1987; Paulsson & Ehrenberg, 1998). Therefore it would be expected that plasmids are generally lost within a few generations, as plasmid-free cells are more competitive within the population. The presence of beneficial genes on the plasmid would be expected to help ensure their propagation under stressful conditions due to the selective advantage to the host cell, but even in the absence of selection, plasmids are frequently retained within the population. This maintenance is so effective that even low-copy-number plasmids can have a loss rate as low as 10^{-4} per generation (Hiraga, 2000).

This high stability cannot be explained by the presence of the basic replicon systems alone. The basic system is not capable of regulating plasmid copy number, which could potentially lead to plasmid-free daughter cells if fewer than normal numbers of plasmid copies are produced during division. In addition, this basic system will only allow for random distribution of plasmid copies, and this cannot ensure efficient plasmid inheritance. Other occurrences can reduce the number of plasmid copies available for distribution between daughter cells. One such phenomenon is dimer catastrophe, a situation where multi-copy plasmids are subjected to homologous recombination within the bacterial host (section 1.8.1). Therefore, the basic replicon must be supported by other systems to achieve high levels of plasmid stability (Nordström & Austin, 1989).

Experiments in the 1970s showed that plasmids contained specific regions of DNA that increased the stability of inheritance. Plasmid loss rates were increased by deletions within the plasmid DNA, suggesting that mechanisms for ensuring stable inheritance were encoded within the genetic structure. In 1974, Yoshikawa defined the *repB* region in plasmid R100 as "not necessary for replication but needed for stable replication". Further work defined several more regions that could stabilise the inheritance of plasmids, such as the *psi* region of plasmid pSC101 (Cornet *et al*., 1994) and the *par* region of plasmid R1 (Jensen *et al*., 1998). Although it was initially thought that all stability regions encoded systems that actively distributed plasmid copies to the daughter cells, now described as true partition systems (section 1.9.4), further analysis suggested there were many other types of system that could increase plasmid stability (Nordström & Austin, 1989).

There are two major groups of plasmid maintenance systems, the helper elements, which reduce the randomness of distribution of plasmids (sections 1.9.1 through 1.9.3), and the true partition systems, which actively separate plasmids into daughter cells, in a similar way to chromosome division (section 1.9.4). High copy-number plasmids generally only require helper elements, as the chance of a plasmid-free cell arising is low because of the large plasmid stock. Low copy-number plasmids generally require a true partition system to ensure that all daughter cells obtain a copy of the plasmid after division.

Plasmid name	Stability system	Function	Reference
P1	*lox-cre*	site-specific recombination	Qian *et al*., 2001
pSC101	*rep*	copy number control	Ingmer *et al*., 2001
pSC101	*par*	control of supercoiling	Hiraga, 1992
R1	*hok-sok*	toxin-antitoxin	Mikkelsen & Gerdes, 1997
F	*sop*	'true' partition	Kim & Shim, 1999

Table 1.2: To show the variety of mechanisms by which plasmid stability can be achieved

1.9.1 Site-specific recombination systems

Oligomer formation during plasmid division can occur frequently due to the numerous copies of identical DNA being produced. Up to 95% of plasmid pACYC184 DNA can be in an oligomeric form in a *rec+* host because of recombination between the homologous plasmid copies (Summers, 1991). Formation of dimers can lead to dimer catastrophe, where dimers replicate more frequently than monomers, leading to a reduced copy number (Boe & Tolker-Nielsen, 1997; Summers, 1998).

Because of this possibility, several plasmids use a site-specific recombination system to increase the copy number by resolving the multimers. In some cases both the recombination site and the recombinase enzyme are plasmid encoded, as, for example, in the *lox-cre* system in plasmid P1 where the Cre recombinase coded for on the plasmid binds to the *loxP* site present on the plasmid (Qian *et al*., 2001). Other plasmids rely on host-encoded genes; for example, ColE1-type plasmids contain the recombination site *cer*, but require the host-encoded Xer recombinase for multimer resolution (Villion & Szatmari, 2003). Plasmids that contain a complete recombination system have often acquired it from a transposon, such as Tn*3*, which requires a system for co-integrate intermediate resolution (Adamczyk & Jagura-Burdzy, 2003).

1.9.2 Systems that control copy number and topology changes

Variance of the copy number of a plasmid must be tightly controlled to ensure accurate

partition (Ingmer *et al*., 2001). Large plasmids generally have a low copy number to prevent a high metabolic burden on the host cell. Plasmid pEB114 is a large cloning vector that replicates to a high copy number within the cell, but this causes a large metabolic burden, resulting in decreased plasmid stability. Mutation of the gene responsible for copy number control (*cop*-1) led to a reduced copy number and resulted in an increased stability, even though the chance of plasmid-free cells arising was increased after cell division (Leonhardt & Alonso, 1988; Leonhardt, 1990). The increased stability was due to the reduction in plasmid burden.

In contrast, smaller plasmids tend to have high copy numbers to ensure that daughter cells receive at least one plasmid copy, as the metabolic burden to the host cell is minimal. Plasmid pAMß1 is a small plasmid with a naturally low copy number that showed instability during cell division. A 100-fold increase in the copy number dramatically increased the stability of this plasmid (Allen & Blaschek, 1990).

ColE1-type plasmids have a copy number control system to ensure that the plasmid will propagate in pace with the host chromosome. This system controls the level of plasmid replication by controlling production of the RNA primers required for initiation of DNA replication (Paulsson & Ehrenberg, 1998; Sozhamannan *et al*., 1999).

Changes in the topology of the plasmid also seem to affect the stability during cell division. Plasmid pSC101 has a region essential for stable plasmid partition termed *par*. The region does not encode a protein, but is a binding site for DNA gyrase, so the stabilising effect may be due to changes in the topology of the DNA (Nordström & Austin, 1989). If the region is mutated the plasmid has a reduced level of supercoiling, and becomes very unstable (Hiraga, 1992). It is not fully understood why supercoiling is important for partition; it may help to reduce plasmid clumping in the cell, expose protein binding sites required for plasmid replication, or allow binding to a host structure as part of a partition mechanism (Manen *et al*., 1990; Miller *et al*., 1990). A model has been devised that allows plasmid copy number and plasmid distribution within the cell to be determined in order to assist in interpretation of the function of the control systems (Greenhalf *et al*., 1989). This assists in interpretation of the copy number control systems.

1.9.3 Killer systems

These systems do not function by ensuring correct plasmid partition during cell division; rather they destroy plasmid-free cells by using a poison and antidote system (Cooper & Heinemann, 2000). These types of systems are not the most efficient plasmid stability mechanism because the growth rate of the population is reduced, although it does ensure that the plasmid is maintained within the population (Nordström & Austin, 1989).

The F plasmid contains the *ccd* system which produces two proteins; CcdB which is toxic to the cell and very stable, and CcdA which is an antidote and labile. While the plasmid remains in the cell the antidote will block the action of the toxin. However, if the plasmid is lost, the antitoxin will quickly degrade, enabling the stable toxin to destroy the cell (Afif *et al*., 2001). In plasmid R1, the *hok* gene encodes a stable toxic protein. The antidote in this case is RNA (*sok*), which is labile. The *sok* RNA is antisense to the *hok* mRNA, and when both are present in the cell, binding of the two RNA molecules will occur, preventing production of the toxin protein (Mikkelsen & Gerdes, 1997).

1.9.4 True partition systems

The term 'true partition system' was first coined by Williams and Thomas (1992) in order to define a system that actively ensured that daughter cells would receive a plasmid copy at cell division. All the systems described in sections 1.9.1 to 1.9.3 function by reducing the chance of plasmid-free cells arising, while true partition systems actively decreases the production of plasmid-free cells.

The majority of low copy-number plasmids contain a true partition system that provides very accurate partition, ensuring that at least one copy of the plasmid is inherited by each daughter cell. The general mechanism is as follows: a plasmid encoded protein attaches to a *cis* acting site on the plasmid; the proteins on two plasmid copies then bind to give paired plasmids; the paired plasmids then bind to a host structure, which assists in segregation of the plasmids into daughter cells (Austin, 1988; Summers, 1991; Hiraga, 2000).

All the plasmids containing true partition (Par) systems seem to contain two genes, which code for *trans*-acting proteins, and one *cis*-acting centromere-like site. These three regions appear to be the essential requirements for true partition. The ParB protein recognises and binds to the *cis*-acting site, and then interacts with the ParA protein whose ATPase activity is responsible for gathering the energy required for partition (Williams & Thomas, 1992; Adamczyk & Jagura-Burdzy, 2003).

For example, the F plasmid codes for two proteins, SopA and SopB, and contains a *cis*-acting site, *sopC* (Niki & Hiraga, 1997; Hirano *et al*., 1998; Kim & Shim, 1999). The structure of the protein-DNA complexes formed during F plasmid partition has been analysed, and it appears that the DNA wraps around the protein, recognising short repeat sequences (Lynch & Wang, 1994). This system does appear to require additional help from the host *E. coli* cell, as loss of certain genes within the host cell chromosome prevents stable maintenance of the F plasmid (Niki *et al*., 1988). A similar system is seen within the plasmid QpH1 of *Coxiella burnetii* (Lin & Mallavia, 1994), and in the virulence plasmid pMT1 of *Yersinia pestis* (Youngren *et al*., 2000).

The segregation of plasmids into daughter cells is not yet fully understood, partly because it appears that several different mechanisms can perform this function (Adamczyk & Jagura-Burdzy, 2003). In some bacteria the plasmids appear to attach to the host chromosome and move with the chromosome to the plane of cell division, other plasmids use host-encoded proteins to attach to the cell membrane and move as the septum is produced between the daughter cells (Miller & Kline, 1979; Watanabe *et al*., 1989; Hiraga, 2000). The growth of cytoskeletal filaments may also be involved in moving the paired plasmids from the centre of the cell to each end before division (Williams & Thomas, 1992).

Techniques have been developed to directly visualise DNA within microbial cells by using fluorescent markers, and this assists in determining the exact method by which partition occurs (Eliasson *et al*., 1992; Gordon *et al*., 1997; Hiraga, 2000). For example, the use of jellyfish green fluorescent protein (GFP) was used to show that the *E. coli* F plasmid migrates to the one-quarter and three-quarter positions within a cell prior to division (Kim & Wang, 1998), and that the R1 plasmid of *E. coli* forms clusters within the cells (Weitao *et al*., 2000).

Regardless of the how the system functions, true partition systems within a plasmid appear to act as incompatibility determinants. If two plasmid species are unable to co-exist within a bacterial population they are described as being incompatible, and this generally means that there is a significant genetic similarity between the plasmids. Therefore, incompatibility between plasmid species containing true partition systems would suggest that there is a high degree of similarity in these systems even though they are found in plasmids from many diverse organisms (Austin & Nördstrom, 1990; Wilson & Figurski, 2002).

1.10 The origins of the plasmid stability systems used in this study

The stability systems analysed in this study are found in two different plasmids, from where they were added into engineered plasmids in order to be used for this work. In order to appreciate the mechanisms of the systems in the engineered plasmids, the original plasmids will be discussed in sections 1.10.1 and 1.10.2. The way in which the stability systems from these plasmids were used in this study will be discussed in section 1.10.3.

1.10.1 The plasmid stability system of prophage P7

Infection of an *E. coli* cell by the bacteriophage P7 results in the formation of a prophage, which is stably maintained in the host cell as a low copy-number plasmid. Complete sequencing of the prophage has recently been completed; the prophage is 102kb in size and appears to contain at least 120 protein encoding genes, organised into 46 operons. It contains two transposable elements, *IS903* and *Tn3*, of which the latter is responsible for conferring ampicillin resistance to the prophage (Łobocka *et al*., unpublished). When the prophage was studied it was seen to contain a 7kb region, where all the information for stable plasmid inheritance is found, which is now termed the P7 *par* region (Ludtke *et al*., 1989; Radnedge *et al*., 1998).

The P7 *par* region encodes two proteins, ParA and ParB, and includes a downstream *cis*-acting site *parS* (Ludtke *et al*., 1989; Radnedge *et al*., 1998). ParB is a DNA binding protein, which binds to the *parS* region in conjunction with the host integration host factor (IHF) protein. It is possible that the IHF protein is responsible for binding

the plasmid to the host membrane. ParA is an ATPase, the activity of which is stimulated when ParB is found bound to the *parS* region. ParA and ParB also function as co-regulators of the *par* operon. ParA represses transcription of the region, and ParB functions to enhance this repression, meaning that high levels of the proteins will reduce further protein production (Radnedge *et al*., 1998).

The P7 partition system is closely related to the partition system of bacteriophage P1, which also includes a *cis*-acting *parS* site and two plasmid-encoded proteins (Martin *et al*., 1987). Similarities in the genetic sequences between the partition systems in P1 and P7 were observed as a result of complete DNA sequencing, and this seems to indicate a common, although distant, ancestor (Ludtke *et al*., 1989; Manen *et al*., 1990).

1.10.2 The plasmid stability systems of plasmid RK2

The IncPα plasmid RK2 contains several mechanisms for ensuring effective plasmid stability, including a true partition system (*ctl*), a post-segregational killing system (*kil*) and a multimer resolution system (*par* / *mrs*) (Adamczyk & Jagura-Burdzy, 2003).

An overview of the structure of plasmid RK2 is given in Fig. 1.3. It is an IncPα plasmid that is over 60kb in size, and encodes at least 74 genes, a summary of which are given in Table 1.3. The genes are tightly packed together within the genome, including some genes that share overlapping reading frames, and 60 of the genes are known to express proteins (Pansegrau *et al*., 1994). It can be seen from Fig. 1.3 that the stability systems of plasmid RK2 occupy a relatively small proportion of the genome.

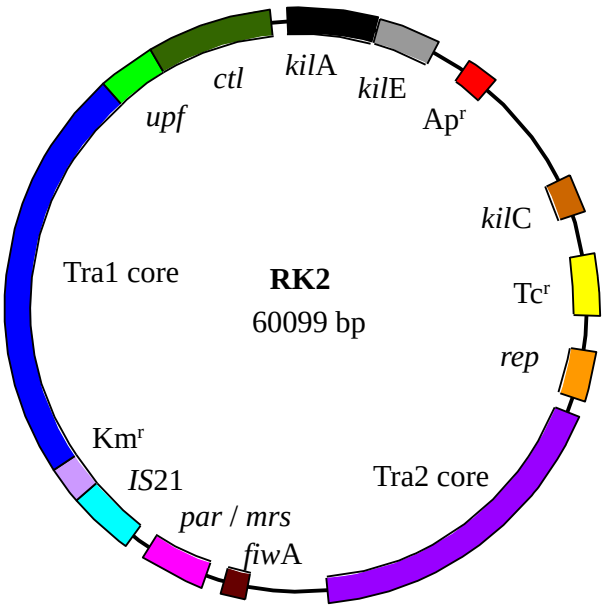


Fig. 1.3: The plasmid RK2 (Pansegrau *et al.*, 1994)

Gene name	**Function**
*kil*A / *kil*C / *kil*E	Regions that produce proteins to create a host killer system
Ap^r / Km^r / Tc^r	Ampicillin, kanamycin and tetracycline resistance respectively
rep	Replication region that includes the gene *trfA* responsible for producing the proteins required to activate the plasmid origin, *ori*V
Tra1 /Tra2 core	Large sets of genes, many of which produce proteins that assist in conjugative transfer of the plasmid
*fiw*A	A gene coding for a protein that prevents plasmids within the IncW incompatibility group from replicating within the host cell.
par/mrs	Stability system that functions through multimer resolution
*IS*21	Transposable element
upf	Genes thought to be involved with partitioning system *ctl*
ctl	Partitioning system that contains *par* genes and a trans-acting *cis* site, as well as other determinants

Table 1.3: Abbreviations for the genes present in plasmid RK2 (shown in Fig. 1.3) (Pansegrau *et al.*, 1994)

The *mrs* system is encoded for by the *parCBA* operon and can function perfectly adequately on its own to produce a reasonable level of plasmid stability (Easter *et al*., 1998). ParA is a resolvase that can mediate site-specific recombination at the multimer resolution (*res*) site. ParB has endo- and exo-nuclease activity and is thought to be involved with recombination and the dissolution of multimers. The function of ParC is as yet unknown, although deletion of the *parC* gene significantly reduces the effectiveness of this stability system (Johnson *et al*., 1999; Adamczyk & Jagura-Burdzy, 2003).

The *parDE* operon is responsible for the *psk* system, the activity of which results in the death of plasmid-free segregants (Roberts & Helinski, 1992). ParE is the lethal polypeptide and appears to function by inhibiting the action of DNA gyrase. The antagonist to this toxin is ParD, which also functions as an autoregulator of the system (Balzer *et al*., 1992; Easter *et al*., 1998; Adamczyk & Jagura-Burdzy, 2003).

Recently, another system known as the *kle/klc* region has been discovered in plasmid RK2, which appears to improve the stability of the RK2 plasmid. Its purpose is not yet fully understood, although it appears to be part of a toxin/antitoxin system (Wilson *et al*., 1997; Thorsted *et al*., 1998; Adamczyk & Jagura-Burdzy, 2003).

It also appears that plasmid RK2 relies on copy number control in order to increase plasmid stability. Replication of the plasmid relies on the action of the plasmid encoded protein TfrA, and of the host provided DnaA protein to initiate handcuffing, a process by which plasmid copies bind to prevent additional replication (Toukdarian & Helinski, 1998; Doran *et al*., 1999).

The region containing the true partition system is described as the *ctl* region. This region is complex and contains many genes as shown in Fig. 1.4 (Williams *et al*., 1998). The genes responsible for providing the true partition system are described below.

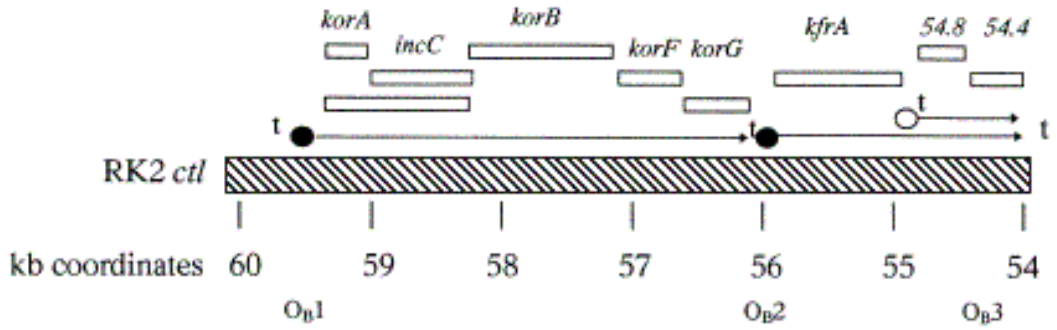


Fig. 1.4: **The RK2 *ctl* region, showing the location of the genes present (Williams *et al*., 1998)**

There are two ParA-like *trans*-acting proteins produced from the RK2 region. These are termed IncC-1 and IncC-2. IncC-2 appears to be responsible for the true partitioning, although IncC-1 is still required, most likely in a regulatory role (Williams *et al*., 1998; Adamczyk & Jagura-Burdzy, 2003). The ParB-like protein in plasmid RK2 is termed KorB, and possesses DNA binding activity. It appears to function as a tetramer *in vivo* (Kornacki *et al*., 1987; Theophilus & Thomas, 1987; Williams *et al*., 1993; Adamczyk & Jagura-Burdzy, 2003). There are twelve potential *cis*-acting sites present in the RK2 region termed O_B1 through O_B12, and the true site has not been categorically identified (Kostelidou & Thomas, 2000; Adamczyk & Jagura-Burdzy, 2003). It appears that O_B3 is the most likely candidate; however there is some indication that O_B1 is also involved (Williams *et al*., 1998). Fig. 1.5 shows the model suggested for the functioning of the partitioning system from plasmid RK2.

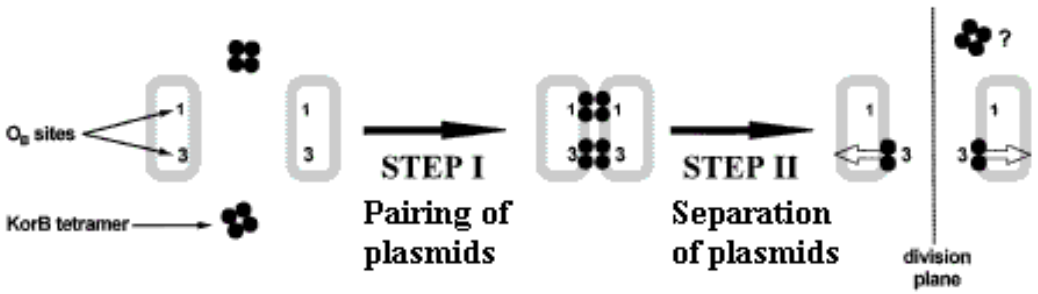


Fig. 1.5: **A suggested model for plasmid partitioning at the *ctl* region of plasmid RK2 (Williams *et al*., 1998)**

The suggested mechanism for the plasmid partitioning of plasmid RK2, shown in Fig. 1.5, is as follows. Two tetramers of KorB protein bind to the O_B1 and O_B3 sites on two plasmid copies with the ATPase activity of IncC-1 providing the energy for the process. The formation of these tetramers has been confirmed by DNAseI and hydroxyl radical footprinting (Williams *et al*., 1993). This leads to the formation of pairs of plasmids, shown as step I in Fig. 1.5. Step II is the separation of these paired plasmids ensuring that one copy of the plasmid is present in each daughter cell after division. This separation is thought to occur at the division plane, and only seems to function correctly when the KorB tetramer is bound to the O_B3 site. Disruption of the O_B3 site has been shown to lead to faulty segregation of the plasmid pairs and therefore a reduction in plasmid stability (Williams *et al.*, 1998).

Other proteins produced from the *ctl* region include KfrA, which contains a DNA-binding domain and an extended coiled-coil domain, suggesting it may function as a force-generating motor protein (Jagura-Burdzy & Thomas, 1992). KorA appears to act in a regulatory capacity (Jagura-Burdzy & Thomas, 1995; Kostelidou *et al.*, 1999; Adamczyk & Jagura-Burdzy, 2003) as it is a global regulator that binds to seven operators, and is responsible for repressing production of itself and KfrA using two of these sites (Jagura-Burdzy *et al*., 1991; Kostelidou *et al*., 1998). KorF and KorG are histone-like proteins that can alter the physical structure of the *cis*-acting site and therefore may carry out a similar function to IHF (Pansegrau *et al*., 1994).

Plasmid RK2 appears to contain an example of almost every type of plasmid stability system so far discovered (Table 1.3). In this study it is the true partition system (found within the *ctl* region) that is of interest. Therefore the plasmids used in this study were engineered to contain only the genes within this region (Williams *et al*., 1998).

1.10.3 The plasmid partition systems used in this study

The *par* system found within the P7 bacteriophage (section 1.10.1) is an effective partition system producing almost complete stability of the plasmid through multiple generations of growth. The genes within the *par* system were transferred to plasmid pBR322 by Ludtke *et al*. (1989) in order to study the structure and function of the system. The resulting plasmid, pALA1029, also showed high levels of stability and was

therefore used in this study as a model of a wild-type stable plasmid.

The *ctl* region of plasmid RK2 (section 1.10.2) was transferred into plasmid pBR322 in order to study its function (Macartney *et al*., 1997; Williams *et al*., 1998). While plasmid RK2 itself is a very stable plasmid, transfer of the *ctl* region alone did not appear to recreate the high levels of stability seen in the parent plasmid. The plasmid containing the complete *ctl* region was named pOG4 and showed a relatively high level of plasmid stability, but it was not inherited as efficiently as pALA1029. Another plasmid engineered in the Williams *et al*. study (1998) was pOG4.003, a plasmid in which the *ctl* region was present, but the O_B3 site was disrupted. This plasmid showed a decreased level of stability. Full details of the plasmids used in this study and their structures are described in detail in chapters two and three of this work.

1.11 Measurement of plasmid stability

The wide variety of plasmid stability systems described in section 1.9 shows that there are plenty of potential targets for new antimicrobial agents. Any of the proteins involved with partitioning could be targeted, and in many cases the alteration or loss of a single protein can cause the downfall of the whole system. In order to determine the effect of a disruption in plasmid partitioning it is necessary to be able to measure plasmid stability.

Techniques for measuring plasmid stability have been used for many studies in the pursuit of several different types of data. One of the main reasons for analysing plasmid stability is in an effort to improve the yields of recombinant proteins in large-scale industrial fermentations. *Lactococcus* strains are essential in the manufacture of cultured milk products, but the genes required for uptake and utilisation of lactose are frequently found on unstable plasmids (Ward *et al*., 1993). By measuring plasmid stability under different growth conditions it is possible to optimise the process to ensure that the highest possible plasmid stability is obtained (Gupta & Mukherjee, 2002). Equally, the desire to understand plasmid stability systems has required determination of a method that allows analysis of the effectiveness of these systems under different conditions (Kim & Blaschek, 1989; Roberts & Helinski, 1992; Easter *et al*., 1998).

In general, plasmid stability is measured under batch culture conditions, the same method which has been used to obtain the data described in chapter four of this work. This is a fast and simple method that allows data to be accumulated rapidly. A more detailed description of this method can be found in section 4.1 of chapter four. Plasmid stability can also be measured under steady-state chemostat culture conditions, although this technique has not been so commonly used (Proctor, 1994). For this study, chemostat culture has a large advantage over batch culture, as it more accurately mimics the conditions found in the human body (Freeman *et al*., 2003). The mathematics of chemostat culture, and the reasons for the use of chemostat culture are detailed in section 5.1 of chapter five.

The final method used for the measurement of plasmid stability was also based on chemostat culture, where washout kinetics were used as a way of determining the effect of plasmid destabilisation on the growth rate of a bacterial population. This technique is described in detail in section 6.1 of chapter six.

1.12 Mathematical modelling of plasmid stability

Mathematical models have been used in conjunction with experimental data for many years. Some of the first models were concerned with the behaviour of contaminants, mutants and mixed cultures within a chemostat system (Powell, 1958; Dykhuizen & Davies, 1980; Dykhuizen & Hartl, 1983; Caulcott, 1984). From this area, mathematical models analysing plasmid instability were developed, where a chemostat containing both plasmid-free and plasmid-bearing cells is a mixed culture in its own right (Cooper *et al*., 1987; Rosenfeld & Grover, 1993; O'Kennedy *et al*., 1995; Paulsson & Ehrenberg, 1998). Alterations and modifications to these models are constantly being published, creating a more accurate analysis tool over time (Yang & Chen, 2003).

Plasmid stability is a term used to describe how efficiently a plasmid is maintained within a population of cells. A stable plasmid can remain within the host cell population for many generations. An unstable plasmid will be lost quickly from the host population, as it is not maintained within the individual cells.

Plasmid instability models allow the nature of the instability to be determined even

before experiments are carried out in a laboratory. The success of these models has been demonstrated by correlation of the expected data with actual data taken from experiments (Cooper *et al*., 1987; Davidson *et al*., 1990; Proctor, 1994). These models can be of great use in analysing plasmid stability data, and may even be of assistance in interpreting unexpected results. Although these models are nonlinear, many use linear regression in order to interpret the data (Dunn *et al*., 1995). It is this basic mathematical model using linear regression (described below) that has been used to interpret data obtained in chapters four, five and seven.

In terms of mathematical modelling, there are two important factors that affect plasmid stability. The first is termed *R*; the rate at which plasmid-free cells are generated from plasmid-bearing cells. The second is $d\mu$; the difference in growth rate between plasmid-free and plasmid-bearing cells. These two factors combine to produce the overall plasmid loss within a bacterial population (Baheri *et al*., 2001). There are three main states of plasmid loss within a population. The first is described as $d\mu \gg R$, a state in which the growth rate difference ($d\mu$) is much greater than the segregational instability (R). This means that very few plasmid-free cells are produced, as the partitioning system is very effective; however, when the plasmid-free cells are produced they have a substantial growth advantage over the host cell and will multiply quickly in relation to the plasmid-bearing cells. This is usually the case for a very stable plasmid that causes a high metabolic burden on the host cell, for example where the plasmid encodes for multiple copies of a large protein such as ß-lactamase (Cooper *et al*., 1987).

The second state is where $|d\mu| \leq R$, or the growth rate difference is of a similar size, or smaller than, segregational instability. In these cases the plasmid is probably lacking an effective partitioning system, and therefore the segregational instability (R) is relatively high. Therefore the increase of plasmid-free cells within the population is due to the relatively high frequency of their production. These plasmid-free cells will multiply of their own accord in the absence of selective pressure, but their comparative rate of multiplication ($d\mu$) is not the dominant factor (Cooper *et al*., 1987).

The third state is where $d\mu < 0$ and $|d\mu| \gg R$. This is the case when the plasmid is not stably maintained, in other words R is relatively large, but the plasmid itself actually confers a growth advantage on the cell, in other words the cells grow more efficiently

when the plasmid is present (the reverse of the plasmid burden effect). A good example of this state is where an unstable plasmid encodes resistance to an antibiotic where the cell population is being grown in the presence of antibiotics. The plasmid would be likely to be lost due to a relatively high segregational instability, but any plasmid-free cells will be incapacitated due to the antibiotic selection. Therefore plasmid-free cells cannot multiply within the culture; the only way in which plasmid-free cells are produced is by segregational instability. In this situation, if the value of *R* is not overwhelmingly large then the proportions of plasmid-free and plasmid-bearing cells within the population will tend towards a constant (Cooper *et al*., 1987; Mosrati *et al*., 1993).

1.13 The role of *Escherichia coli* as a model organism

The main reason for the use of *Escherichia coli* C2110 (Table 2.1) in this study was because earlier work in this area has been carried out with this organism and therefore the plasmids used were designed for replication in this particular host (Williams *et al*., 1998). However, although *E. coli* C2110 is non-pathogenic it still retains many similarities to pathogenic strains and this allows certain conclusions to be drawn.

Most infections caused by *E. coli* are fairly harmless in healthy individuals. In many cases these infections will resolve themselves without the use of antibiotics. However, those most at risk from *E. coli* infections are the old and the very young, especially those who have been hospitalised. It is also a major cause of concern in immunocompromised patients where the risk of infection is greatly increased (Jawetz *et al*., 1987; Sleigh & Timbury, 1993). Many *E. coli* infections still require treatment through antibiotics, including long-term urinary tract infections and severe cases of diarrhoea (Shanson, 1989; Sleigh & Timbury, 1993). As these infections become increasingly likely to be caused by antibiotic resistant organisms, those patients most at risk from *E. coli* are also harder to treat through conventional means.

Clinical strains of *E. coli* have been seen to contain antibiotic resistance to almost all widely available antibiotics (Widemann & Grimm, 1996) and therefore results obtained in this study may be directly relevant to clinical infections. Equally, the length of time required for cell division, the growth requirements and the effects of antibiotics will

generally be the same for both the *E. coli* C2110 strain used in this study and clinical *E. coli* isolates.

CHAPTER TWO – MATERIALS AND METHODS

2.1 Bacterial strains

The two bacterial strains used in this study are described in Table 2.1.

Strain designation	Genotype	Origin
E. coli C2110	*polA1 his rha* $P2^S$	Professor D. Helinski, Department of Biology, UC San Diego, CA, USA
E. coli DH5α	*endA1 hsdR17*($r_K^- m_K^-$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1*Δ*(lacIZYA-argF) U169 deoR* (ϕ80*dlac*Δ*(lacZ)*M15)	Dr. Vickers Burdett, Department of Microbiology and Immunology, Duke University, Durham, NC, USA

Table 2.1: The strains of *Escherichia coli* used in this study

The plasmids pALA1029, pOG4, pOG04, pOG4.003, pKO1029, pKO4, pKO04 and pKO4.003 (Table 2.2) each contain two replication origins. The pMB1 origin from pBR322 allows the plasmids to replicate to a high copy number in the $PolA^+$ strain *E. coli* DH5α, from which stocks of plasmid DNA can be extracted. The pMB1 origin does not function in the $PolA^-$ strain *E. coli* C2110; instead replication proceeds from the P7 origin from bacteriophage P7. This origin controls plasmid replication, ensuring that plasmids are kept at a low copy number, which allows the stability of the plasmids to be tested in their most unstable state in terms of copy number. A key for the abbreviations used in Table 2.1 can be found in appendix A.

2.2 Plasmids

The plasmids used in this study are listed in Table 2.2.

Plasmid name	Reference	Fig.
pBR322	Bolivar *et al*., 1977	2.1
pALA1029	Ludtke *et al*., 1989	2.2
pOG4	Macartney *et al*., 1997	2.3
pOG04	Macartney *et al*., 1997	2.4
pOG4.003	Williams *et al*., 1998	2.5
pJL3-1974	Machida *et al*., 1983	2.6
pKO1029	Oliver, unpublished	2.7
pKO4	Oliver, unpublished	2.8
pKO04	Crewe, unpublished	2.9
pKO4.003	Oliver, unpublished	2.10
pOG004	Williams, unpublished	2.11

Table 2.2: The plasmids used in this study

Figs. 2.1 to 2.11 can be found on the following pages. A key for the abbreviations used in Figs. 2.1 to 2.11 can be found in appendix B, and a summary of the construction of each plasmid is given below the diagram.

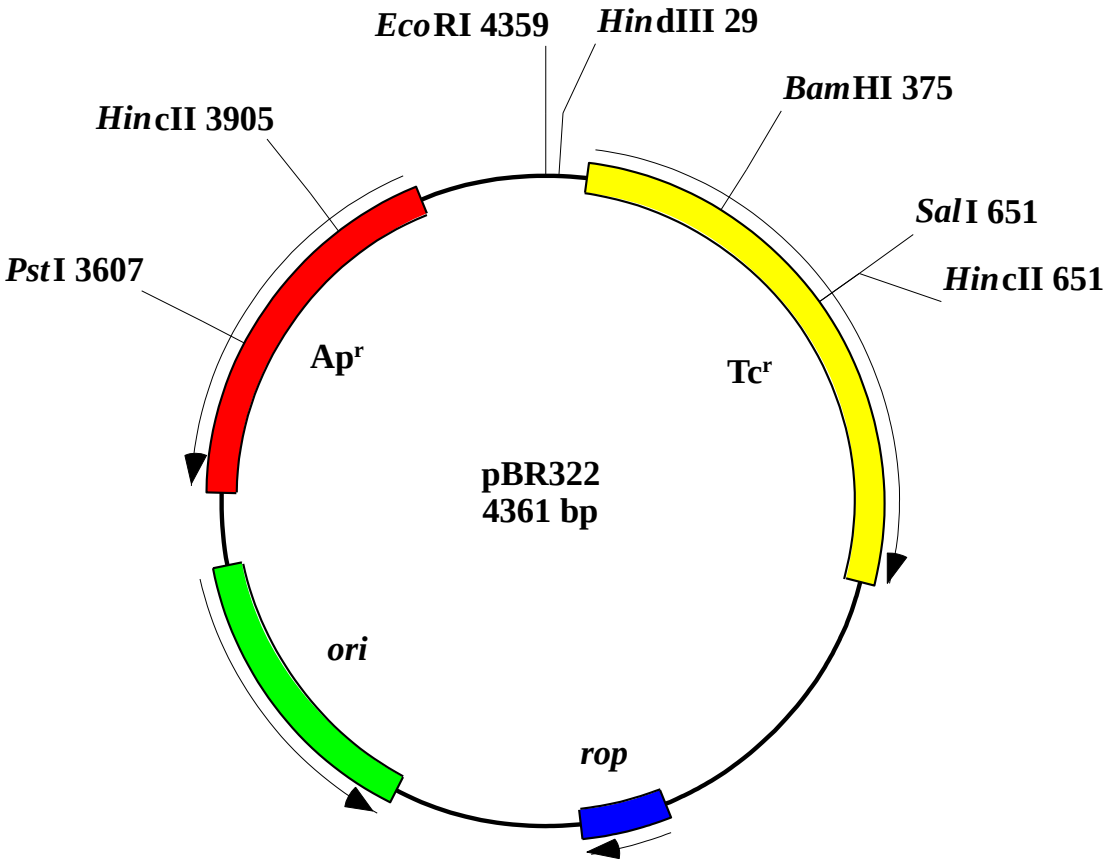


Fig. 2.1: The plasmid pBR322

Plasmid pBR322 was constructed in 1977 for use as a new cloning vector. The design was engineered in order to improve on the structure of its parent plasmid, pBR313 (Bolivar *et al*., 1977), which was originally constructed from pSC101 (Cohen *et al*., 1973). pBR322 contains a single *Pst*I site in the centre of the gene for ampicillin resistance, and also contains several other unique restriction sites. As pBR322 is a small plasmid it is possible to insert relatively large amounts of DNA into the backbone without significantly reducing the efficiency of replication. The origin of replication (originally from plasmid pMB1, a plasmid in the ColE1 compatibility group) allows the plasmid to replicate to a high copy number in a PolA$^+$ cell (Bolivar *et al*., 1977).

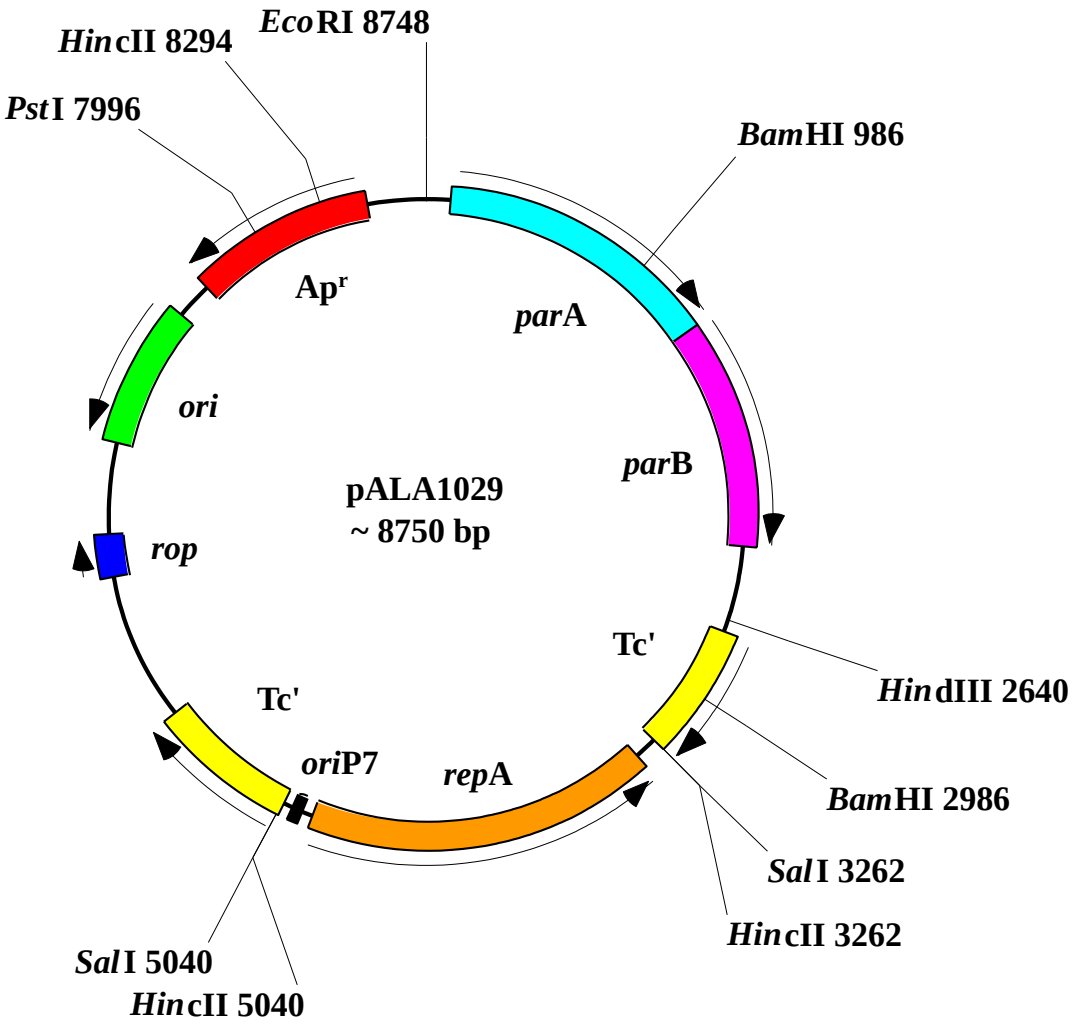


Fig. 2.2: The plasmid pALA1029

pALA1029 was constructed in order to study the *par* stability system present in bacteriophage P7. The *par*A and *par*B genes were cloned between the *Eco*RI and *Hin*dIII sites of plasmid pBR322. The region for replication of P7 (*ori*P7 and *rep*A) was cloned into the single *Sal*I site of pBR322. As a result resistance to tetracycline is disrupted. When this plasmid is transformed into a PolA$^+$ strain replication proceeds from the pMB1 origin resulting in a high copy number. Replication in a PolA$^-$ strain proceeds from the P7 origin, resulting in a low copy number (Ludtke *et al.*, 1989).

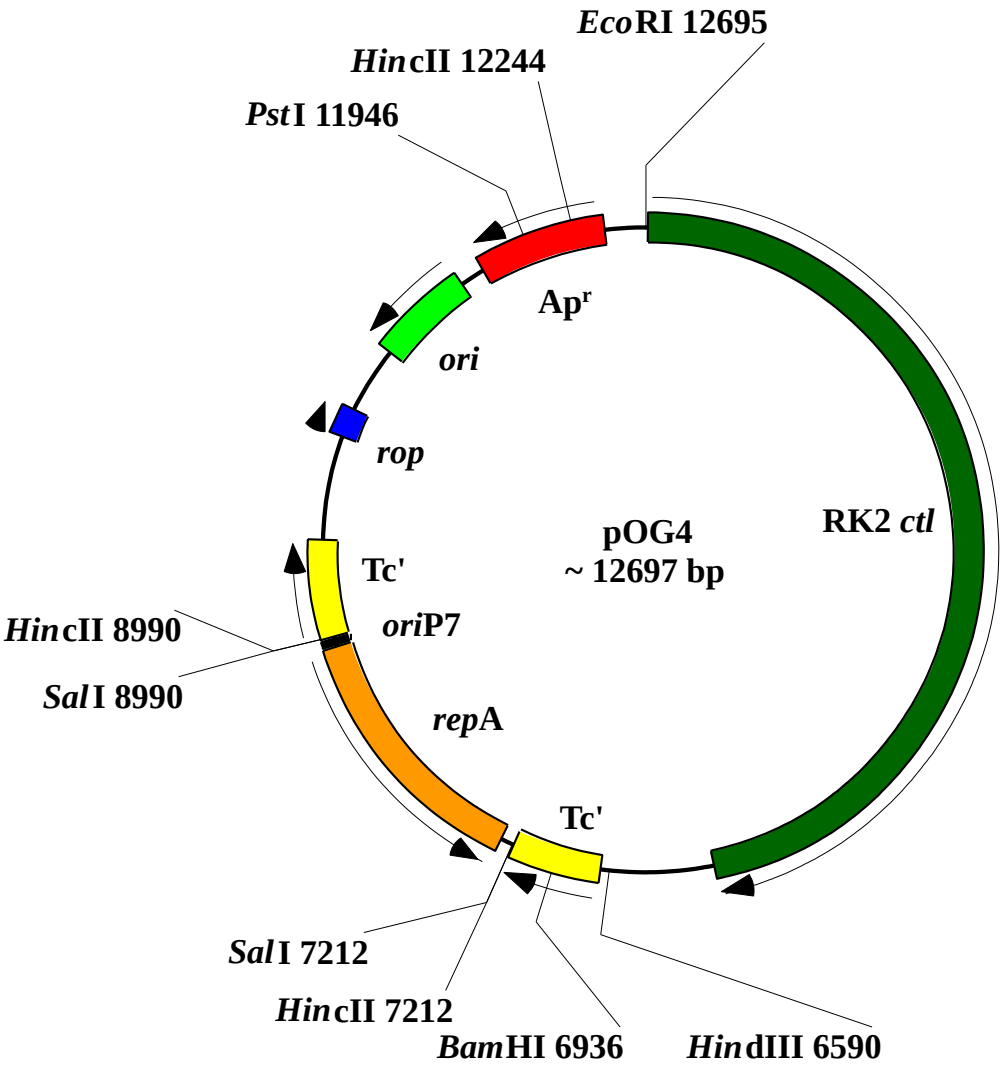


Fig. 2.3: The plasmid pOG4

Plasmid pOG4 was derived from pALA1029 in order to test the stability system present in plasmid RK2. The *par*A and *par*B genes present between the *Eco*RI and *Hin*dIII sites in pALA1029 were replaced with the *ctl* region from plasmid RK2. Otherwise the plasmid remains unchanged from that of pALA1029 (Macartney *et al*., 1997).

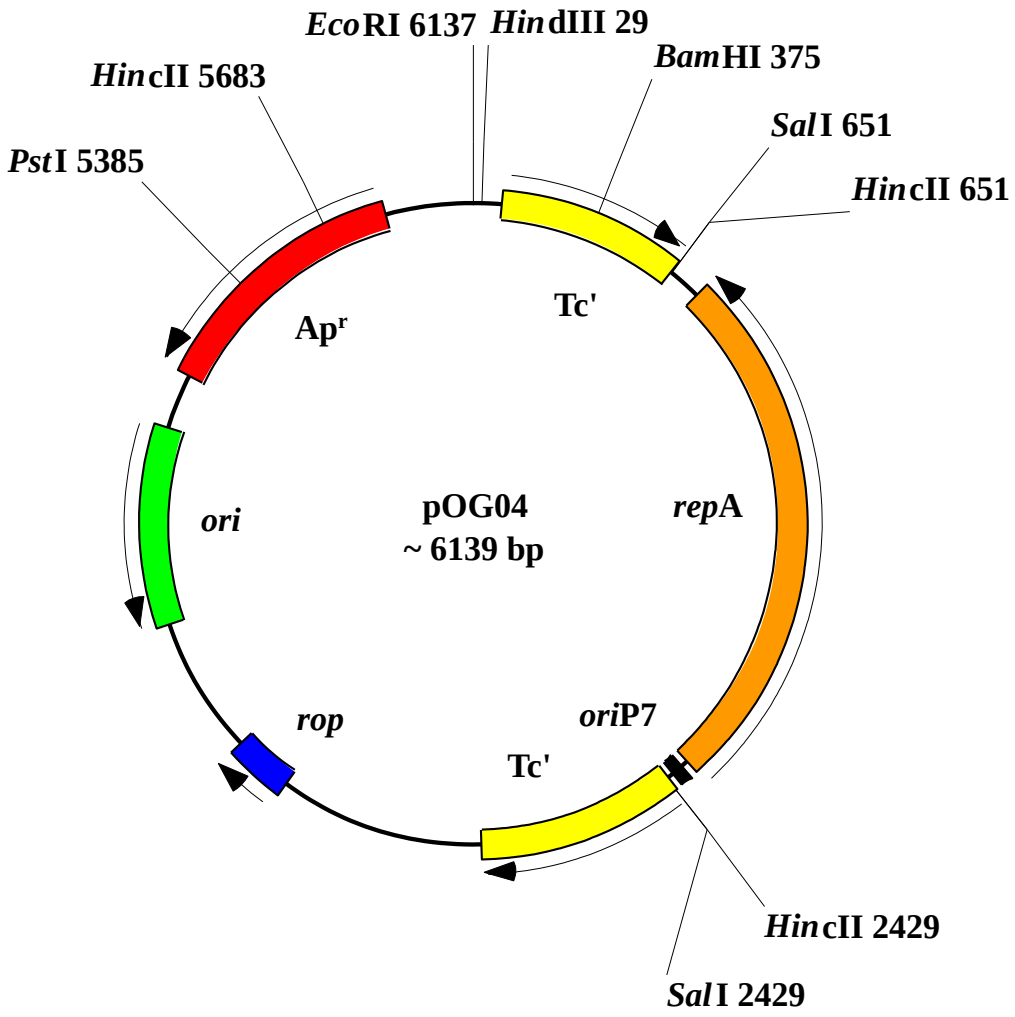


Fig. 2.4: The plasmid pOG04

pOG04 was constructed as a control plasmid for the work on stability systems carried out by Macartney *et al*. (1997) and Williams *et al*. (1998). Plasmid pALA1029 is the parental plasmid; the *par*A and *par*B genes present between the *Eco*RI and *Hin*dIII sites were removed and replaced with the original 32 base pair sequence from pBR322 (Macartney *et al*., 1997). This plasmid contains no stability system and as a result is subject to random segregation during replication. It does however still contain the *ori*P7 and *rep*A regions allowing replication at a low copy number in a PolA$^-$ host.

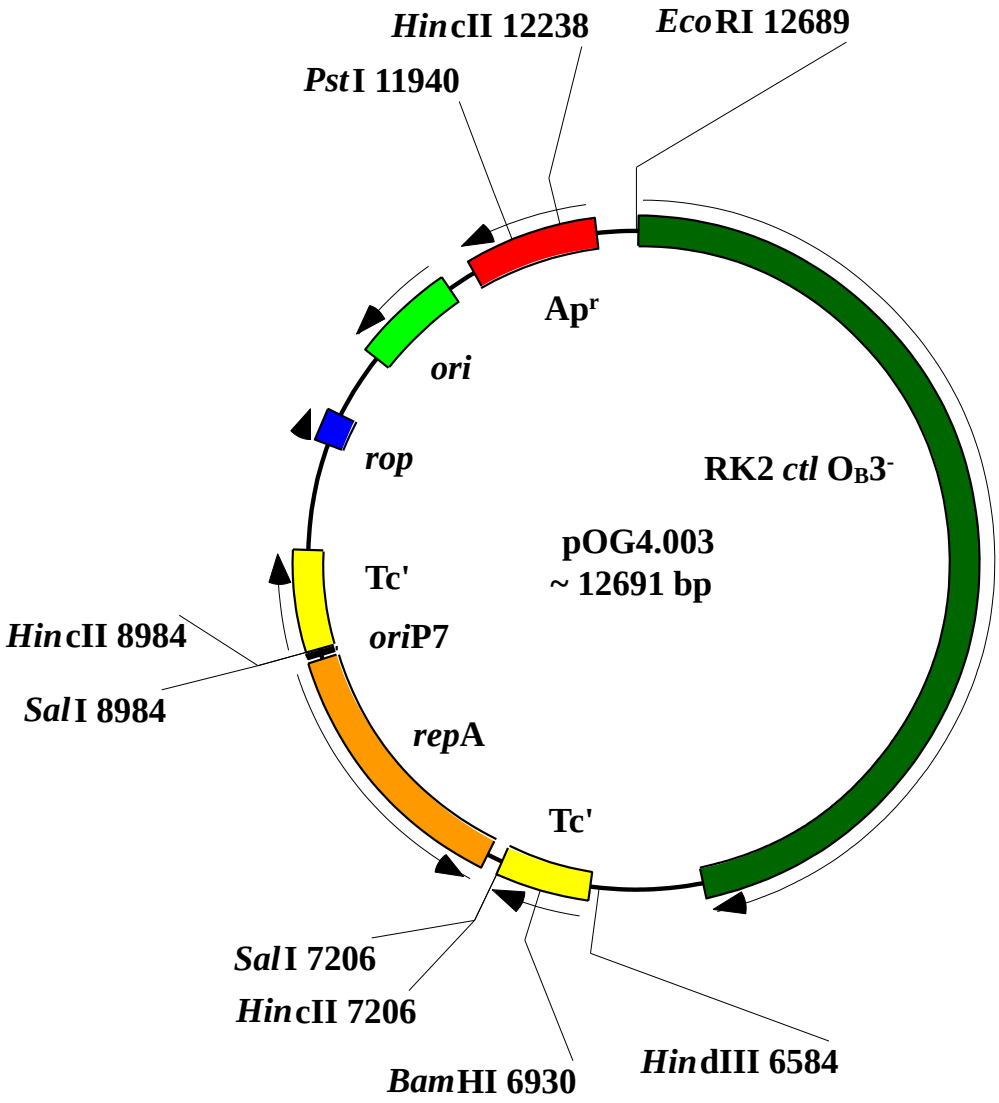


Fig. 2.5: The plasmid pOG4.003

Plasmid pOG4.003 was derived from plasmid pOG4. In pOG4.003 the O_B3 site present in the *ctl* region has been subjected to site-directed mutagenesis and as a result is no longer functional. This plasmid was one of a series produced by Williams *et al*. (1998) in order to determine the function of the genes and sites present in the RK2 *ctl* region. When tested, plasmid pOG4.003 was seen to be unstable in terms of plasmid segregation, suggesting that the O_B3 site was of high functional importance.

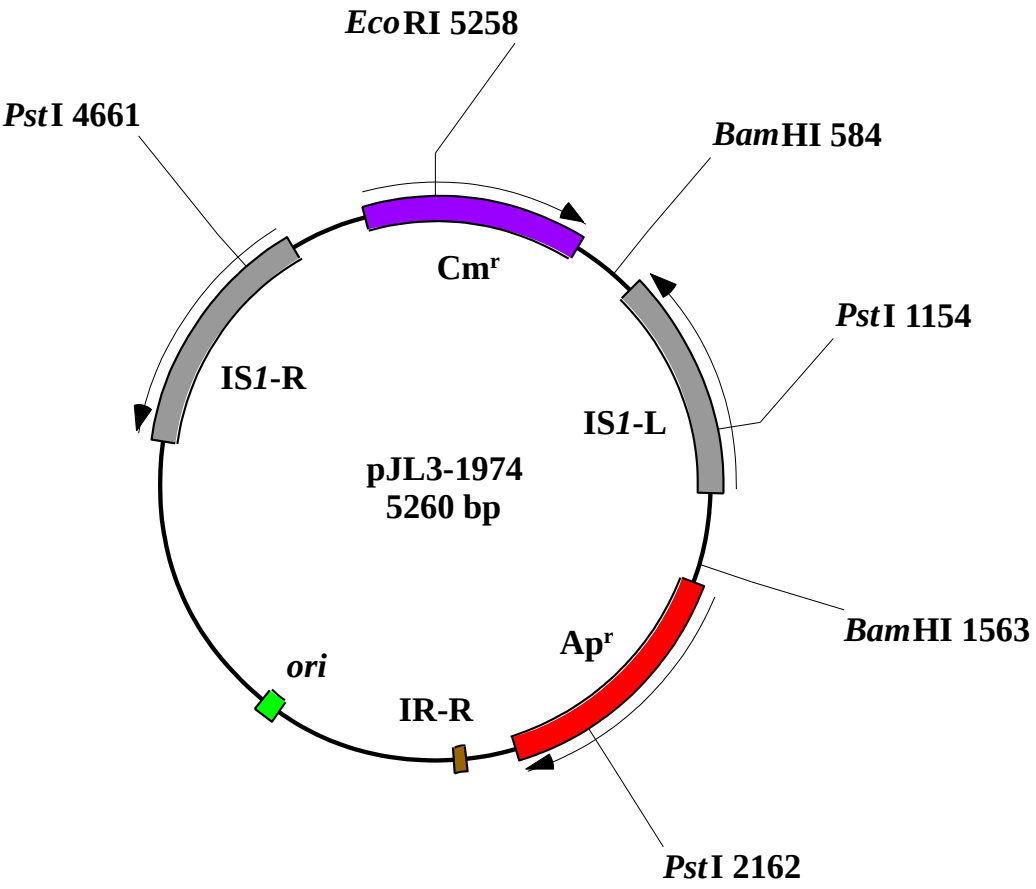


Fig. 2.6: The plasmid pJL3-1974

Plasmid pJL3-1974 was constructed by Machida *et al*. (1983) in order to study the function of insertion element IS*1*. For the present study it was used as a source of the chloramphenicol acetyl transferase gene. The plasmid was digested using *Pst*I in order to extract the fragment of DNA containing the chloramphenicol acetyl transferase gene, and this fragment was inserted into pALA1029, pOG4, pOG04 and pOG4.003 using the single *Pst*I site in the centre of the ß-lactamase gene (chapter three).

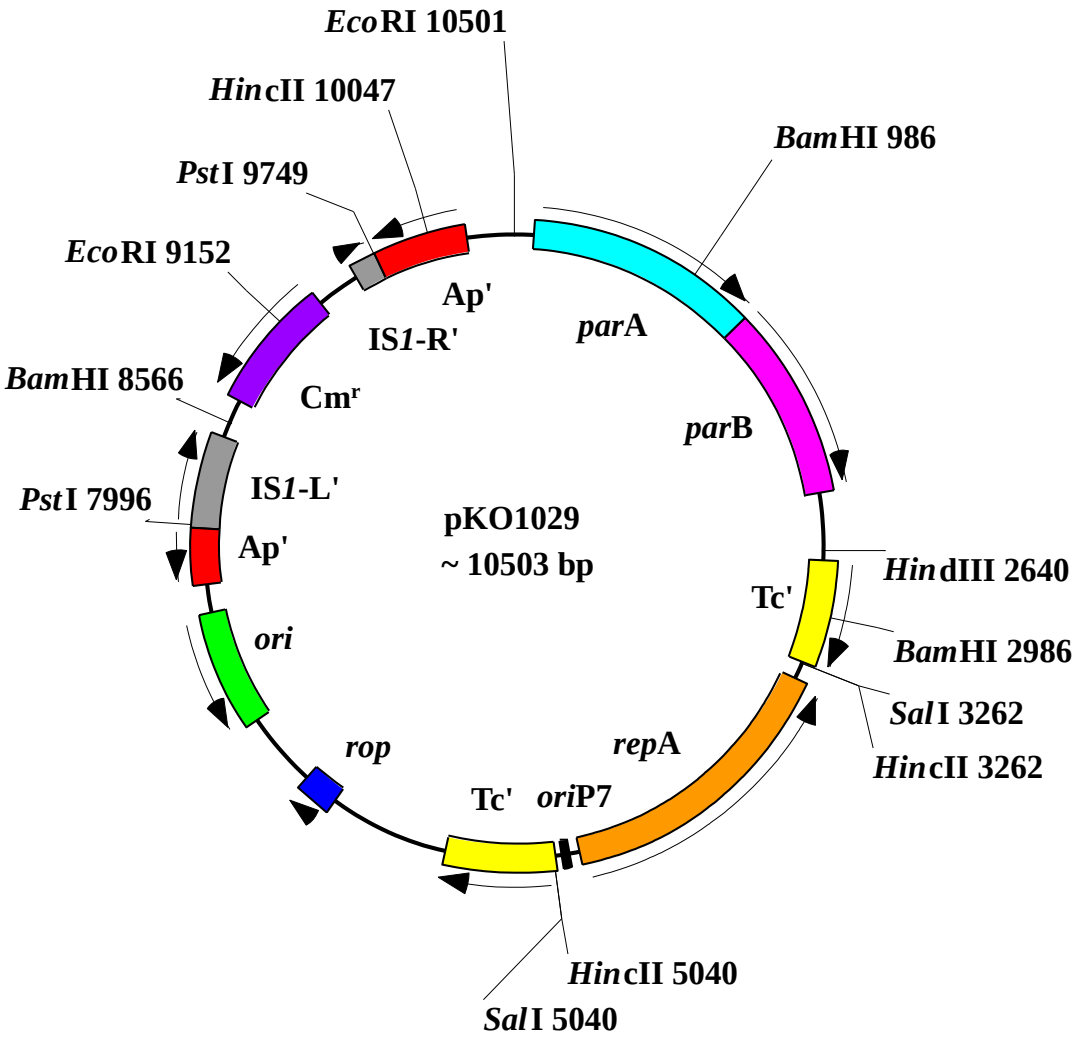


Fig. 2.7: The plasmid pKO1029

Plasmid pKO1029 was constructed from plasmid pALA1029 by insertion of the chloramphenicol-resistance gene from pJL3-1974 into the *Pst*I site present in the ampicillin-resistance gene (Oliver, unpublished). As a result, resistance to ampicillin is removed and replaced by resistance to chloramphenicol. This plasmid was constructed in order to test the effect of a change in antibiotic resistance on the stability of the plasmid (chapter three).

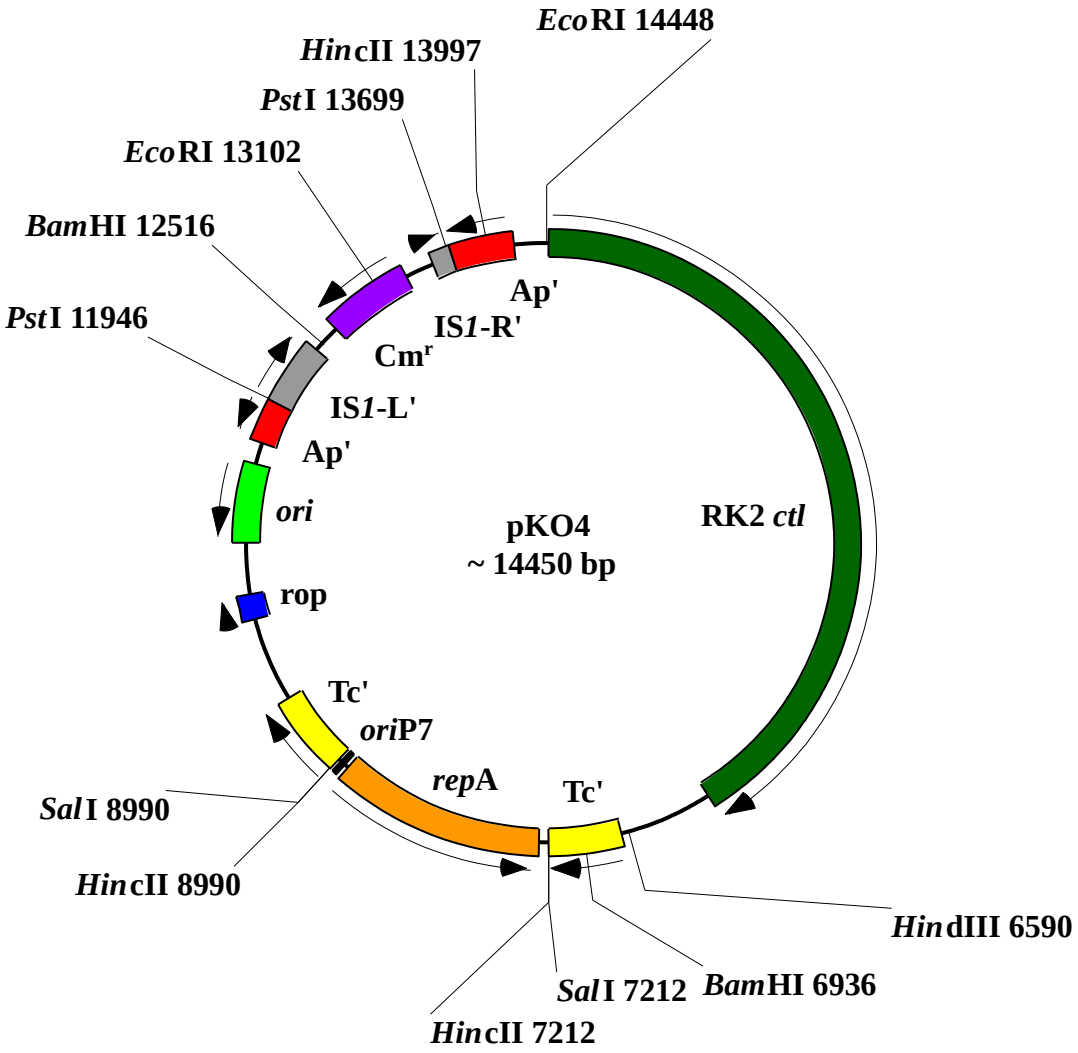


Fig. 2.8: The plasmid pKO4

Plasmid pKO4 was constructed from plasmid pOG4 by insertion of the chloramphenicol-resistance gene from pJL3-1974 into the *Pst*I site present in the ampicillin-resistance gene (Oliver, unpublished). Further details of the procedure are given in chapter three.

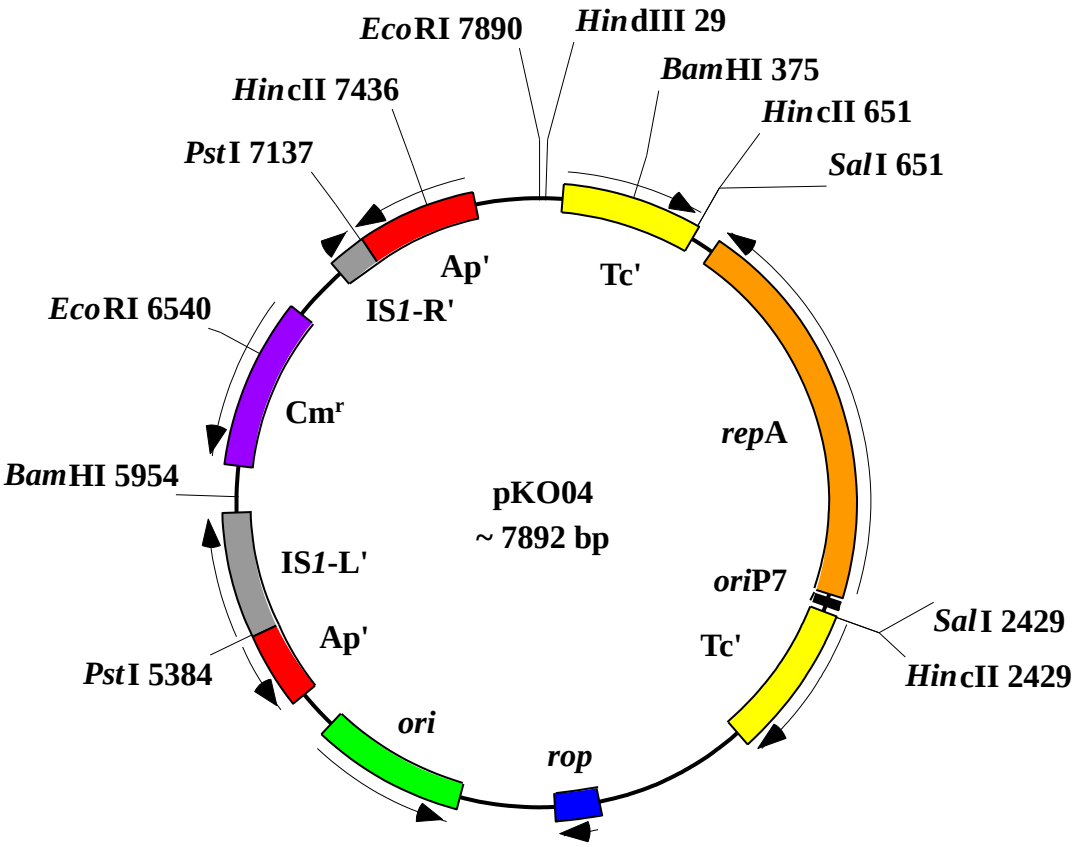


Fig. 2.9: The plasmid pKO04

Plasmid pKO04 was constructed from plasmid pOG04 by insertion of the chloramphenicol-resistance gene from pJL3-1974 into the *Pst*I site present in the ampicillin-resistance gene. Further details on the procedure are given in chapter three.

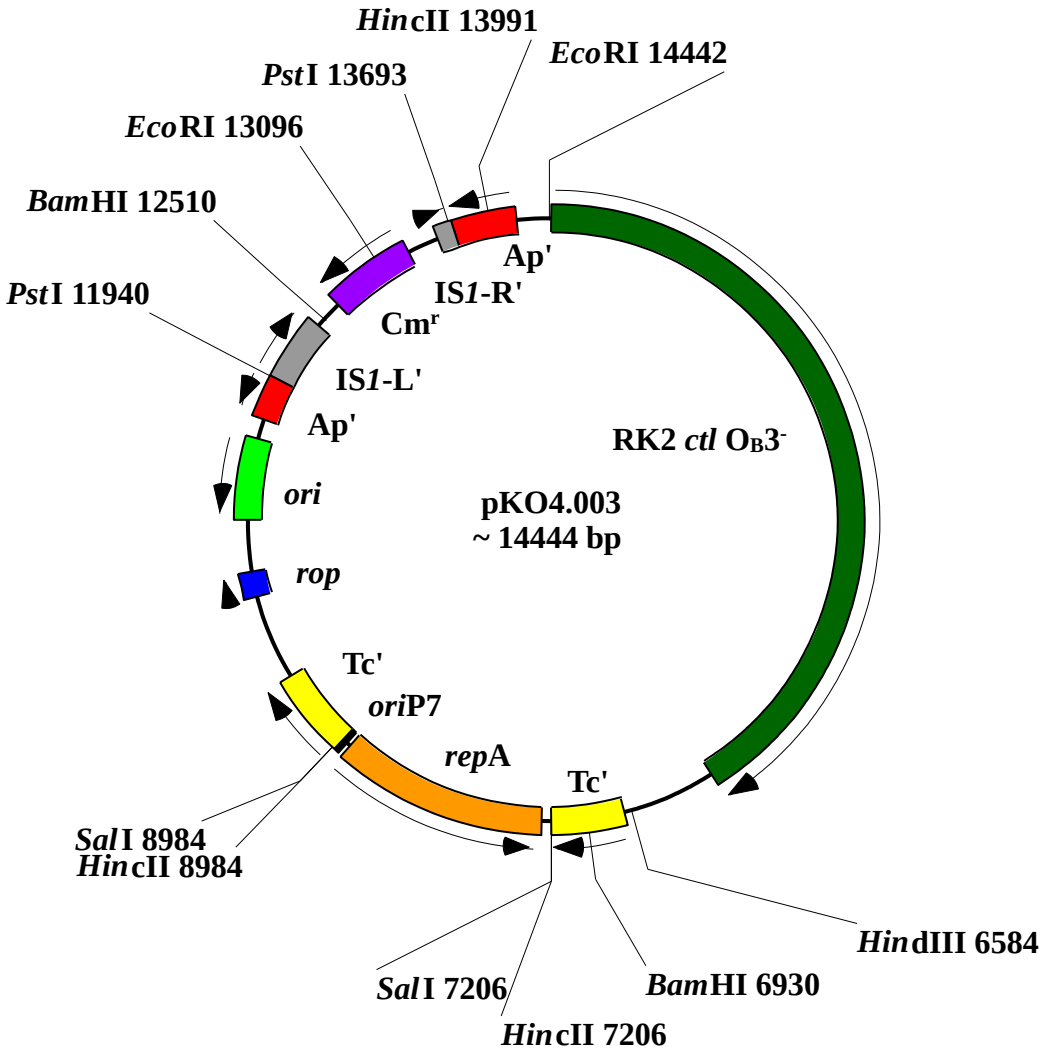


Fig. 2.10: The plasmid pKO4.003

Plasmid pKO4.003 was constructed from plasmid pOG4.003 by insertion of the chloramphenicol-resistance gene from pJL3-1974 into the *Pst*I site present in the ampicillin-resistance gene (Oliver, unpublished). Further details on the procedure are given in chapter three.

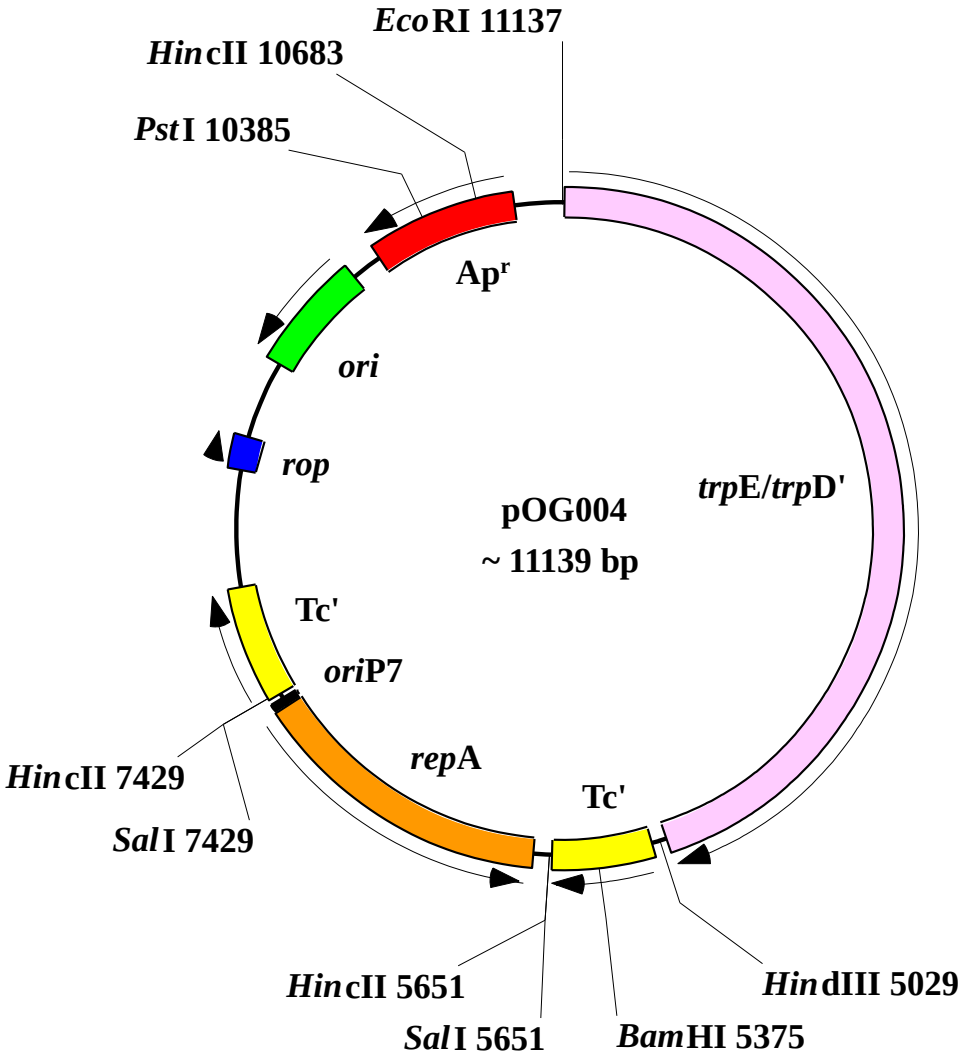


Fig. 2.11: The plasmid pOG004

Plasmid pOG004 was constructed from pOG04 to be used as a control plasmid for stability experiments (Williams, unpublished). The *trp*E gene and the truncated *trp*D gene from plasmid pRK353 (Kolter & Helinski, 1978) were inserted into the *Eco*RI to *Hin*dIII region of pOG04. The *trp*E and *trp*D genes were originally obtained from a fragment of the *E. coli* genome. In the present study pOG004 was used as a probe for Southern blotting. Further details are given in section 2.12.4 and in chapter three.

2.3 Media and supplements

2.3.1 Luria-Bertani Medium

The complex medium used throughout for growth of *E. coli* in batch culture was Luria-Bertani (LB) medium (Bertani, 1951). In the absence of agar the medium was termed LB broth. When agar was added to the medium it was used to produce LB agar slopes, LB agar stabs or LB agar plates.

Tryptone	10 g l^{-1}
NaCl	10 g 1^{-1}
Yeast extract	5 g l^{-1}
Agar bacteriological no. 1	1% (w/v) added for solid media

Half-strength LB broth was used for the fermenter-based culture to establish substrate limitation.

Tryptone	5 g l^{-1}
NaCl	5 g l^{-1}
Yeast extract	2.5 g l^{-1}

2.3.2 Antibiotic supplements (Williams, D.R. 1998, pers. comm.)

Stock solutions of ampicillin and chloramphenicol were produced at the following concentrations.

Ampicillin	40 mg ml^{-1} dissolved in MilliQ water
Chloramphenicol	25 mg ml^{-1} dissolved in a mixture of 50% MilliQ water and 50% ethanol.

The stock solutions were sterilised by filtration with a 0.2 µm sterile syringe filter and stored at 4°C for a maximum of one month. For supplementation of solid media the molten agar was allowed to cool to 50°C before addition of the antibiotic to prevent decomposition. Solid media supplemented with antibiotics were stored for a maximum of one week at 4°C. Liquid media were allowed to cool completely before addition of

antibiotic, and were only supplemented immediately before use, to ensure full effectiveness of the antibiotic.

2.4 Storage and resuscitation of *E. coli* C2110 and DH5α

2.4.1 Initial resuscitation and subsequent storage of *E. coli* C2110 and DH5α (Williams, D.R. 1998, pers. comm.)

The initial source of *E. coli* strains C2110 and DH5α were from stabs in LB agar (section 2.3.1) in bijou bottles. Cells were aseptically transferred to 25 ml of LB broth (section 2.3.1) for overnight growth at 37°C with shaking (250 rpm). Single colonies were isolated from the overnight culture, and one single colony was sub-cultured into 25 ml of LB broth (section 2.3.1) for overnight growth at 37°C. This overnight culture was used to produce fresh stabs in LB agar (section 2.3.1) for use throughout this study. The phenotype of the culture was checked by Gram staining and observation of colony morphology. If further stabs were required, the initial stab was used again as a source of cells to prevent excessive subculturing.

2.4.2 Production of stocks of *E. coli* C2110 and DH5α for regular use (Williams, D.R. 1998, pers. comm.)

Cells were aseptically removed from a stab and transferred to a flask containing 25 ml of LB broth (section 2.3.1) for overnight growth at 37°C. Single colonies were obtained from the overnight culture and one colony was used to inoculate an LB agar slope (section 2.3.1), which was incubated overnight at 37°C. The slope was subsequently stored at 4°C and used as a cell stock for a month, after which time it was discarded and a new slope produced.

2.5 Extraction of plasmid DNA from *E. coli* C2110 or DH5α (adapted from Sambrook *et al.*, 1989)

2.5.1 Solution 1 – resuspension buffer

50 mM glucose,
25 mM Tris-Cl brought to pH 8.0 with 1 M HCl,
10 mM EDTA brought to pH 8.0 with 1 M NaOH.

2.5.2 Solution 2 – lysis solution

0.2 M NaOH,
1% SDS.

2.5.3 Solution 3 – denaturation solution

3 M sodium acetate brought to pH 5.0 with glacial acetic acid.

2.5.4 TE buffer

10 mM Tris-Cl brought to pH 8.0 with 1 M HCl,
1 mM Na_2EDTA brought to pH 8.0 with 1 M NaOH.

2.5.5 Alkaline lysis method

A single colony of *E. coli* containing the plasmid required was selected and transferred to a flask containing 25 ml of LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}. The flask was incubated overnight at 37°C with vigorous shaking. 1.5 ml of the overnight culture was transferred to an Eppendorf tube and centrifuged at 15,000 **g** for 2 minutes; the remainder of the culture was stored at 4°C until the integrity of the extracted plasmid had been verified by gel electrophoresis (section 2.7). The supernatant was discarded and the pellet was partially dried by shaking off any excess liquid. The bacterial pellet was resuspended in 100 µl of ice-cold resuspension buffer (section 2.5.1) by vortexing. 200 µl of lysis solution (section 2.5.2) was added and mixed by gently inverting the tube. 150 µl of denaturation solution (section 2.5.3) was added, the tube was vortexed for 10 seconds, and then placed on ice for 5 minutes. The tube was then centrifuged for 5 minutes at 15,000 **g**. Following this, the supernatant was transferred to a fresh Eppendorf tube and the pellet

was discarded. Two volumes of isopropanol were added, the tube was vortexed to mix the contents and left to stand at room temperature for 2 minutes. The tube was then centrifuged at 15,000 **g** for 5 minutes. The supernatant was discarded and the pellet fully dried by standing the tube upside down on absorbent paper. 1 ml of 70% ethanol was added to the tube and the pellet was resuspended by vortexing. The tube was centrifuged at 15,000 **g** for 5 minutes. The supernatant was discarded and the pellet was fully dried. The pellet was redissolved in 50 µl TE buffer (section 2.5.4) by brief vortexing and the plasmid DNA solution was stored at 4°C.

2.6 Digestion of plasmid DNA with restriction enzymes

The restriction enzymes used during this study were *Eco*RI, *Hin*cII, *Hin*dIII and *Pst*I. (MBI Fermentas). Digests were carried out under the conditions recommended by the supplier; in general the reaction was set up as follows. 1 µl of 10X enzyme specific restriction buffer was added to a small Eppendorf tube and diluted with 7 µl of sterile distilled water. One unit of restriction enzyme was added to the buffer (where one unit is defined as the amount of enzyme required to digest 1 µg of DNA in one hour at 37°C). 1 µg of DNA was added to the tube and mixed, and the tube was placed in a 37°C incubator for 1 hour. After this time the reaction was stopped by the addition of 2 µl of mini-gel stop mix (section 2.7.1) and the samples were loaded on a mini-gel for analysis by 0.8% agarose gel electrophoresis (section 2.7).

2.7 Agarose gel electrophoresis (adapted from Sambrook *et al*., 1989)

2.7.1 Mini-gel stop mix

20% (w/v) sucrose,
10% (w/v) ficoll,
10 mM EDTA,
0.25% (w/v) bromophenol blue.

2.7.2 TAE buffer

0.04 M Tris brought to pH 8.0 with glacial acetic acid,
0.001 M EDTA brought to pH 8.0 with 1 M NaOH.

2.7.3 Method for agarose gel electrophoresis

DNA was digested with restriction enzymes (section 2.6). A 0.8% (w/v) agarose gel was prepared with TAE buffer (section 2.7.2), and allowed to set in a mini-gel former. Once set, the gel was placed into the mini-gel apparatus and submerged in TAE buffer (section 2.7.2). The restricted DNA samples containing mini-gel stop mix were loaded onto the gel and the electrophoresis was allowed to proceed at 100 V for approximately 1 hour. A staining tank was prepared containing 250 ml of TAE buffer (section 2.7.2) and ethidium bromide at a concentration of 1 µg ml^{-1}. The gel was placed in this staining tank for 15 minutes to allow staining of the DNA to occur. Following this, the gel was placed in a fresh tank containing 250 ml TAE buffer (section 2.7.2) for destaining. The gel was then visualised using a gel documentation system containing a UV transilluminator (302 nm) (GelVue) linked to a computer based analysis system (Gene Genius, Bio Imaging System, Syngene).

2.8 Production of competent cells of *E. coli* DH5α and C2110 (adapted from Sambrook *et al*., 1989)

Cells from the stock agar slope (section 2.4.2) were used to inoculate 5 ml of LB broth (section 2.3.1), and grown overnight at 37°C with agitation. After overnight growth, 0.1 ml of the culture was transferred to a conical flask containing 50 ml of LB broth (section 2.3.1). The flask was incubated at 37°C with vigorous shaking until OD_{600} ~ 0.35, and was placed on ice for 10 minutes to cool the culture to 0°C to prevent further cell growth. The culture was then transferred aseptically to a 50 ml polypropylene centrifuge tube, and the cells were harvested by centrifugation at 6,000 **g** for 10 minutes. The supernatant was removed and discarded and 10 ml of ice-cold sterile 0.1 M $CaCl_2$ was added to the cell pellet. The pellet was resuspended by vortexing, and the tube was placed on ice for 30 minutes. The cells were harvested by centrifugation at 6,000 **g** for 10 minutes. The supernatant was removed and discarded and 4 ml of ice-cold sterile 0.1 M $CaCl_2$ was added to the tube. The pellet was resuspended by vortexing and the tube was placed on ice. The cells were then competent and capable of taking up plasmid DNA. The competent cells could be used immediately for transformation (section 2.9) or could be stored overnight at 4°C and used the following day.

2.9 Transformation of competent bacterial cells with plasmid DNA (adapted from Sambrook *et al*., 1989)

200 µl of competent cells (section 2.8) were transferred into a sterile 1.5 ml Eppendorf tube. To this tube, approximately 0.2 µg of plasmid DNA was added and gently mixed. The tube was placed on ice for 30 minutes and then transferred to a heating block at 42°C for 90 seconds. The tube was then immediately placed on ice for 2 minutes. 800 µl of LB medium (section 2.3.1) was added to the tube, and the tube was placed in a 37°C incubator for 45 minutes to allow recovery of the cells and expression of the antibiotic resistance marker encoded by the plasmid. 100 µl of the transformed cell mixture was spread onto an LB agar plate (section 2.3.1) containing ampicillin at 50 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}. The plate was incubated overnight at 37°C.

2.10 Extraction of DNA from an agarose gel

A Geneclean® II kit (BIO101) was used to extract high purity DNA from an agarose gel for cloning (chapter 3) and probe production for Southern blotting (section 2.12.4).

The DNA was digested (section 2.6) and then run on an agarose gel (section 2.7). After staining with ethidium bromide the gel was placed on a UV-emitting light box (302 nm) to allow visualisation of the DNA bands. The bands of DNA required were cut out of the gel and placed in a sterile Eppendorf tube. The weight of the agarose gel in the tube was determined, and 1 ml of a 3 M NaI solution was added per 1 g of agarose gel. The tube was placed in a waterbath at 50°C, and mixed occasionally until the agarose had melted. 5 µl of Glassmilk® (BIO101 - crystalline silica (SiO_2) suspended in water) was added to the tube and mixed gently for 5-10 minutes. The tube was centrifuged at 15,000 **g** for 5 seconds, and the supernatant was discarded. The pellet was resuspended in 500 µl of NEW Wash® (BIO101 - NaCl, Tris and EDTA dissolved in an ethanol and water mixture - commercial product), and the tube was centrifuged at 15,000 **g** for 5 seconds. The supernatant was discarded. The wash step was repeated a further three times. After the final wash the pellet was dried to remove all traces of the NEW Wash® solution. 50 µl of TE buffer (section 2.5.4) was added to the tube and the pellet was resuspended. The tube was centrifuged at 15,000 **g** for 5 seconds. The supernatant

after this centrifugation step contained approximately 80% of the DNA that was present in the agarose gel. This proportion of DNA was sufficient for the Southern blotting and cloning procedures.

2.11 Ligation (adapted from Sambrook *et al*., 1989)

Equimolar concentrations of the vector DNA and foreign DNA were placed in an Eppendorf tube. 7.5 µl of sterile distilled water was added, and the mixture was warmed to 45°C to melt any cohesive termini that had reannealed. The mixture was then chilled to 0°C. 1 µl of 10X T4 ligation buffer (MBI Fermentas) and 0.1 of a Weiss unit of bacteriophage T4 DNA ligase (MBI Fermentas) were added (where 0.015 Weiss units is defined as the amount of enzyme that will ligate 50% of the *Hin*dIII fragments of 5 µg of bacteriophage λ DNA in 30 minutes at 16°C). The reaction was left to proceed at room temperature for four hours. The ligated DNA was used to transform competent DH5α cells (sections 2.8 and 2.9).

2.12 Estimation of relative plasmid copy number (Williams *et al*., 1998)

Relative plasmid copy number was determined for the plasmids pKO1029, pKO4, pKO04 and pKO4.003 in *E. coli* C2110 (chapter three). The following sections describe the methods used.

2.12.1 Total DNA extraction

Competent *E. coli* C2110 cells (section 2.8) were transformed (section 2.9) with one of the plasmids pKO1029, pKO4, pKO04 or pKO4.003 and plated onto LB agar (section 2.3.1) containing 10 µg ml^{-1} of chloramphenicol. After overnight growth, a transformed colony was transferred to a flask containing 25 ml of LB broth (section 2.3.1) containing 20 µg ml^{-1} of chloramphenicol and incubated at 37°C with vigorous shaking until OD_{600} ~ 1.0. 0.75 ml of this culture was placed in a 1.5 ml sterile Eppendorf tube. 0.75 ml of a phenol/chloroform/isoamyl alcohol solution (25:24:1 buffered with 100 mM Tris and 1 mM EDTA, pH 8.0) was added to the cell culture. The tube was mixed by vortexing for approximately 30 seconds, and then was centrifuged for 5 minutes at 15,000 **g**. The aqueous (top) layer was transferred to a sterile Eppendorf

tube. The bottom (organic) layer was discarded. A further 0.75 ml of phenol/chloroform/isoamyl alcohol was added to the aqueous solution. The tube was mixed by vortexing for approximately 30 seconds, and then was centrifuged for 5 minutes at 15,000 **g**. The aqueous layer was transferred to a sterile Eppendorf tube, ensuring that no phenol/chloroform/isoamyl alcohol was carried over. One volume of isopropanol was added to the tube, which was mixed and left at room temperature for 2 minutes. The tube was centrifuged for 5 minutes at 15,000 **g**, the supernatant was removed and discarded, and 1 ml of ice-cold 70% (v/v) ethanol was added to the pellet. The tube was mixed by vortexing in order to dislodge and clean the pellet and then centrifuged for 5 minutes at 15,000 **g**. The supernatant was removed and discarded, and the pellet was dried and resuspended in 50 µl of TE buffer (section 2.5.4).

2.12.2 Determination of DNA concentration and gel electrophoresis of total DNA extracts

In order to ensure that a clear Southern blot is produced, it is important to use a standard concentration of DNA in all lanes run on the gel. As a result, the concentrations of the total DNA extracts were determined using spectrophotometric analysis. Dilutions of each of the total DNA extracts were made and absorbance readings were taken at 260 nm and 280 nm. From these readings the amount of protein contamination and the concentration of DNA in the extracts was determined.

2 µg of DNA from each of the total extractions were digested with restriction enzyme *Eco*RI (section 2.6), and run on a 0.8% agarose gel (section 2.7). An *Eco*RI digest (section 2.6) of plasmid pOG004 (Fig. 2.11) was also run on the gel in order to provide a control for the probe. After staining with ethidium bromide the gel was then briefly visualised using a gel documentation system containing a UV transilluminator (302 nm) linked to a computer based analysis system to ensure DNA was present in the gel. The gel was then used in the Southern blotting procedure (section 2.12.3).

2.12.3 Southern blotting (adapted from Sambrook *et al*., 1989; except section 2.12.4)

2.12.3.1 Denaturation solution

1.5 M NaCl,

0.5 M NaOH.

2.12.3.2 Neutralisation buffer

1.5 M NaCl,

0.5 M Tris-Cl brought to pH 7.2 with 1 M HCl,

0.001 M EDTA.

2.12.3.3 20X SSPE blotting buffer (diluted 10-fold to produce 2X SSPE and 20-fold to produce 1X SSPE)

3.6 M NaCl,

0.2 M Sodium phosphate buffer,

0.02 M EDTA brought to pH 7.7 with 1 M NaOH.

2.12.3.4 Setting up the Southern blotting apparatus

Once the gel had been visualised (section 2.12.2) it was soaked in sufficient denaturation solution (section 2.12.3.1) to cover the gel, and left for 30 minutes at room temperature. The denaturation solution was poured away and the gel was rinsed with distilled water. The water was poured away and the gel was soaked in enough neutralisation buffer (section 2.12.3.2) to completely cover the gel. The gel was left for 15 minutes at room temperature with occasional shaking. The neutralisation buffer was then poured away, fresh neutralisation buffer was added to completely cover the gel, and the gel was left for 15 minutes at room temperature with occasional shaking. The southern blotting apparatus was then assembled.

A large tray was filled with 20X SSPE blotting buffer (section 2.12.3.3). A platform was placed in the buffer. Three large sheets of Whatman 3MM filter paper were placed over the platform, long enough to touch the blotting buffer to form a wick. These 3MM

sheets were then saturated with 20X SSPE blotting buffer. The agarose gel was placed on the 3MM paper and any bubbles were removed by rolling a glass test tube gently over the gel. The gel was surrounded with clingfilm to prevent a 'shortcut' where the blotting buffer could be directly absorbed into the paper towels above. A sheet of Hybond-N membrane the exact size of the gel was cut and slowly rolled onto the surface of the gel. Any bubbles were removed by rolling a glass test tube gently over the membrane. Three sheets of Whatman 3MM paper were cut to the same size as the gel and placed on top of the membrane. A stack of absorbent paper towels approximately 5 cm high were placed on top of the 3MM paper. A glass plate was placed on top of the paper towels and a 1 kg weight was balanced on top.

Once the apparatus had been assembled, the transfer of the DNA proceeded overnight. The apparatus was carefully dismantled and the membrane was marked to allow identification of the DNA side and orientation of the lanes of the gel. The membrane was briefly washed in 2X SSPE (section 2.12.3.3) to remove any adhering agarose and then baked at 80°C for 2 hours to fix the DNA. The membrane was subsequently stored in a sealed plastic bag.

2.12.4 Probe preparation (Williams *et al*., 1998)

In order to determine the relative plasmid copy number, a probe that would bind to both chromosomal and plasmid DNA was required. Plasmid pOG004 (Fig. 2.11) contains the *E. coli* chromosomal gene *trp*E as well as the backbone found in all eight of the stability plasmids being used in this study. This plasmid would therefore bind to both the chromosome and whichever of the plasmids was present in the cell at the time of the total DNA digest.

Plasmid pOG004 was digested with *Eco*RI (section 2.6) and run on a 0.8% agarose gel (section 2.7). Several wells within the gel were combined to allow a large volume of plasmid DNA to be extracted. After staining with ethidium bromide the single band of DNA was extracted using the Geneclean® procedure (section 2.10) to give purified plasmid DNA. The concentration of the DNA was calculated by spectophotometric analysis and the solution was diluted with TE buffer (section 2.5.4) to give a final concentration of 28 ng μl^{-1}.

140 ng of *Eco*RI-digested pOG004 DNA was placed in a sterile Eppendorf tube to act as the template DNA. 75 ng of random primers (6 base pair random oligomers) was added to the tube and mixed. The tube was heated to 100°C for 5 minutes to cause denaturation of the template DNA, and then cooled to allow primer annealing. To this tube was added reaction buffer (final concentration of 50 mM Tris-Cl at pH 8.0, 5 mM $MgCl_2$ and 1 mM DTT); 1 unit of *E. coli* Klenow fragment from DNA polymerase I (where one unit is defined as the amount of enzyme required to incorporate 10 nM of deoxyribonucleotides into a polynucleotide fraction in 30 minutes at 37°C); dATP, dGTP and dTTP (final concentrations of 0.25 mM) and 21 µl of sterile distilled water. 80 µCi of α32P dCTP (specific activity of 3000 Ci mM^{-1}) was added to the tube and mixed. The tube was incubated at 37°C for 10 minutes, and then a final concentration of 1 mM EDTA was added to stop the enzyme reaction. All components of the DNA labelling system were obtained from MBI Fermentas.

The labelled probe was separated from unincorporated dCTP by separation through a mini Sephadex G50 column. The column was primed with TE buffer (section 2.5.4), and allowed to equilibrate. 50 µl of the labelled probe mixture was added to the column. The column was continuously filled with fresh TE buffer (section 2.5.4) to ensure it did not dry out. Two drops of the eluted liquid were collected in 20 consecutive Eppendorf tubes. The radioactivity of each tube was measured using a Geiger counter, and the tubes containing the first large radioactive peak were pooled to give approximately 300 µl of radiolabelled probe.

2.12.5 Pre-hybridisation

25 ml of Amersham ECL gold hybridisation buffer (commercial product) was placed in a flask with 1.25 g of Amersham blocking reagent (commercial product) and 0.73 g of NaCl to give a final concentration of 0.5 M in the buffer. The buffer was added to the plastic bag containing the nylon membrane (section 2.12.3.4) and incubated for 1 hour at 65°C with shaking.

2.12.6 Hybridisation

The probe (section 2.12.4) was heated at 100°C for 5 minutes to cause denaturation.

1ml of pre-hybridisation buffer was removed from the bag containing the nylon membrane (section 2.12.5) and added to the denatured probe. This was mixed and then returned to the bag. Hybridisation was allowed to proceed at 65°C overnight with shaking.

2.12.7 Washing

Following hybridisation, the buffer and probe were carefully disposed of, and the nylon membrane was washed with 2X SSPE (section 2.12.3.3) containing 0.1% (w/v) sodium dodecyl sulphate (SDS). The membrane was left at room temperature for 10 minutes. The wash solution was removed and replaced with fresh solution, and the membrane was left at room temperature for a further 10 minutes. The wash solution was removed and replaced with 1X SSPE (section 2.12.3.3) containing 0.1% (w/v) SDS, and the membrane was incubated for 15 minutes at 55°C. The wash solution was then removed and replaced with fresh solution, and the membrane was incubated for a further 10 minutes at 55°C.

2.12.8 Autoradiography

The membrane was removed from the washing solutions and wrapped in clingfilm. The nylon membrane was placed in a sealed cassette with photographic film for 1 week and then the film was developed by usual methods.

2.13 Culture of micro-organisms (Williams *et al*., 1998)

Several different methods of micro-organism culture were used during this study. All these methods are described in detail below. For each experiment involving culture of *E. coli* C2110 containing a plasmid, a fresh transformant was used. This enabled the cultures to be traced and helped to prevent mutation through excessive subculturing. At the end of each experiment, plasmid DNA was extracted from a colony and analysed as described below to determine whether structural changes had taken place. This procedure is described below.

A single antibiotic resistant colony from a replica plate produced at the end of the experiment was transferred to a flask containing 25 ml of LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}. The flask was incubated overnight at 37ºC with vigorous shaking. The plasmid was extracted (section 2.5) used to transform (section 2.9) competent *E. coli* DH5α cells (section 2.8). After overnight growth, a single transformant colony was transferred to a flask containing 25 ml of LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}. The flask was incubated overnight at 37ºC with vigorous shaking. The plasmid was extracted (section 2.5) and the plasmid preparation was digested with restriction enzyme *Hin*cII (section 2.6). A sample of the stock plasmid preparation was also digested with *Hin*cII. Gel electrophoresis of the samples was carried out on a 0.8% agarose gel (section 2.7), and after staining the banding patterns were compared to determine whether any gross structural changes had occurred within the plasmid.

2.13.1 Batch culture (chapter four) (adapted from Macartney *et al.*, 1997)

The plasmid to be tested was transformed (section 2.9) into competent *E. coli* C2110 (section 2.8). After overnight growth, a single transformant colony was transferred into a flask containing 25 ml of LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}, pre-warmed to 37°C. The flask was incubated at 37°C with vigorous shaking until OD_{600} ~ 1.0 to generate the inoculum for the experiment. The cells were diluted to $10^{-5.5}$ using a sterile 0.85% (w/v) NaCl solution. 0.1ml of the 10^{-5} and $10^{-5.5}$ dilutions were spread-plated onto LB agar (section 2.3.1) without antibiotic and incubated at 37°C overnight. 250 µl of the 10^{-4} dilution was transferred to the flask to be used for the experiment, which contained 25 ml of fresh LB broth (section 2.3.1) without antibiotic, pre-warmed to 37°C. The flask was incubated overnight at 37°C with vigorous shaking to allow approximately 25 generations of growth. After overnight growth the cells were diluted to $10^{-6.5}$ using a sterile 0.85% (w/v) NaCl solution. 0.1 ml of the 10^{-6} and $10^{-6.5}$ dilutions were spread plated onto LB agar (section 2.3.1) without antibiotic and incubated at 37°C overnight.

Following overnight growth of the spread plates, the number of colonies present on the plates were counted and recorded. The number of colonies present on the first set of

spread plates was used to calculate the CFUs per ml in the flask at the start of the experiment, described as t_o. The number of colonies present on the second set of spread plates was used to calculate the CFUs per ml in the flask at the end of the experiment, described as t_{end}.

The plates with approximately 100 colonies were used for replica plating onto LB agar (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}, and also onto LB agar (section 2.3.1) without antibiotic. The replica plates were incubated overnight at 37°C. After overnight growth of the replica plates, the number of colonies resistant to antibiotic on the replica plate were counted and recorded.

The apparent percentage loss of the plasmid was calculated using the following formulae.

$$L \text{ (loss rate)} = 1 - R^{\frac{1}{g}} \times 100$$

Where $$g \text{ (generations)} = \frac{\ln\left(\frac{t_{end}}{t_o}\right)}{0.693}$$

And $$R = \frac{\%\ Ap^r \text{ or } Cm^r \text{ bacteria at } t_{end}}{\%\ Ap^r \text{ or } Cm^r \text{ bacteria at } t_o}$$

2.13.2 Preparation and inoculation of a chemostat culture (chapters five and seven) (adapted from Corchero & Villaverde, 1998)

The fermenter used in this study was the BioFlow model, produced by New Brunswick Scientific. The fermenter, which had a capacity of 2 litres, contained 1 litre of medium. All fermentations were carried out at 37°C, with agitation at 450 rpm, and pH control at 6.8 by the automatic addition of 0.5 M HCl. Reservoirs containing 10 litres of medium were connected up to the fermenter as required.

2.13.3 Steady state chemostat culture (chapter five) (Williams, D.R. 1998, pers. comm.)

The plasmid to be studied was transformed (section 2.9) into competent *E. coli* C2110 (section 2.8). After overnight growth, a single transformant colony was transferred to a flask containing 25 ml of LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}. The flask was incubated at 37°C with vigorous shaking until OD_{600} ~ 1.0. The culture was used to inoculate a fermenter (section 2.13.2). The fermenter was run in batch culture until OD_{600} ~ 1.0. At this point a medium reservoir of half strength LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1} was connected up to the fermenter vessel. The dilution rate was set to 0.2 hr^{-1}. A sample of approximately 5 ml was immediately taken (t_o). The time of sampling was recorded as 0 hours, the OD_{600} was measured and the cells were diluted to 10^{-5} with sterile 0.85% (w/v) NaCl solution. 0.1 ml of the 10^{-4} and 10^{-5} dilutions were spread plated onto LB agar (section 2.3.1) without antibiotic and incubated at 37°C. Following overnight growth, the number of colonies present on the plates were counted and recorded. The plate with approximately 100 colonies was used for replica plating onto LB agar (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1} and also onto LB agar (section 2.3.1) without antibiotic. The replica plates were incubated overnight at 37°C. After overnight growth of the replica plates, the number of colonies sensitive to antibiotic on the replica plate were counted and recorded. Four further samples (t_1 to t_4) were taken at approximately 19, 43, 67 and 91 hours after the flow was switched on and treated in the same way as the t_o sample.

2.13.4 Washout culture (chapter seven) (adapted from Esener *et al.*, 1981)

Although all washout experiments were carried out in generally the same way, each different set of experiments required alterations to the basic method. In order to clearly describe these changes, the basic method is described below, and each subsequent section will detail the changes made to this method.

1. A flask containing 25 ml of LB broth (section 2.3.1) was inoculated with a single colony from a spread plate.

2. The flask was incubated at 37°C with vigorous shaking until OD_{600} ~ 1.0, and the contents were then used to inoculate the fermenter vessel (section 2.13.2).
3. The fermenter was run in batch culture until OD_{600} ~ 1. 0.
4. A media reservoir containing half-strength LB broth (section 2.3.1) was then connected to the fermenter vessel.
5. The dilution rate was set close to μ_{max} (as determined from the preliminary experiments described in section 2.13.4.1).
6. The fermenter culture was allowed to equilibrate and reach steady state, defined as no change in OD_{600} readings from four samples taken every 15 minutes.
7. Once steady state had been reached the dilution rate was increased to 2.4 h^{-1} to start washout.
8. A sample of approximately 5 ml was taken immediately after the dilution had been increased. The time was noted, OD_{600} was measured and the sample was diluted to 10^{-6} with sterile 0.85% (w/v) NaCl solution. 0.1 ml of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread plated onto LB agar (section 2.3.1) without antibiotic and incubated at 37°C overnight.
9. Further samples were taken every 10 minutes for a total of 110 minutes during the washout experiment and were treated identically to the first sample.
10. Following overnight growth of the spread plates, the number of colonies present on the plates were counted and recorded.
11. The plate with approximately 100 colonies was used for replica plating onto LB agar (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1} and also onto LB agar (section 2.3.1) without antibiotic. The replica plates were incubated overnight at 37°C. After overnight growth of the replica plates, the number of colonies sensitive to antibiotic on the replica plate were counted and recorded.

2.13.4.1 Preliminary experiments - Determination of D_{crit}

The fermenter culture was set up as described in 2.13.4, as far as step 4, with alterations depending on which culture was being tested (as described in sections 2.13.4.2 to 2.13.4.5). As no information of the μ_{max} of these cultures was available, an approximate dilution rate was chosen based on the known division rates of *E. coli*. OD_{600} readings

were taken every 15 minutes to determine whether the biomass within the fermenter vessel was remaining constant or washing out. If the OD_{600} was falling rapidly, then the dilution rate was reduced. If the OD_{600} remained constant then the dilution rate was increased. D_{crit} was determined in this manner by being the dilution rate at which cells started to washout.

2.13.4.2 Washout of *E. coli* C2110 in the absence of antibiotic

The experiments were carried out as described in 2.13.4 with the following alterations:

1. The single colony was that of *E. coli* C2110 with no plasmid.
11. This step was not carried out, as all cells would have shown antibiotic sensitivity.

2.13.4.3 Washout of *E. coli* C2110 in the presence of antibiotic

The experiments were carried out as described in 2.13.4 with the following alterations:

1. The single colony was that of *E. coli* C2110 with no plasmid.
7. A new medium reservoir, supplemented with ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}, was prepared. The dilution rate was increased to 2.4 h^{-1} and the medium reservoirs were swapped over, so that the antibiotic supplemented medium was being supplied to the vessel. Antibiotic was added to the fermenter to instantly raise the concentration to 100 µg ml^{-1} for ampicillin or 20 µg ml^{-1} for chloramphenicol.
8. As cell death was rapid under these conditions, dilutions were altered to ensure colonies were present on the plates after overnight growth.
11. This step was not carried out, as all cells would have shown antibiotic sensitivity.

2.13.4.4 Washout of *E. coli* C2110 containing a plasmid in the absence of antibiotic

The experiments were carried out as described in 2.13.4 with the following alterations:

1. The single colony was a transformant (section 2.9) of *E. coli* C2110 containing the plasmid being studied. This colony was transferred to a flask containing 25 ml of

LB broth (section 2.3.1) containing ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1}.

7. Altered as described in 2.13.4.3.

2.13.4.5 Washout of *E. coli* C2110 containing a plasmid in the presence of antibiotic

The experiments were carried out as described in 2.13.4 with the following alterations:

1. Altered as described in 2.13.4.4
2. The fermenter vessel was supplemented with ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1} prior to inoculation.
4. The medium reservoir was supplemented with ampicillin at 100 µg ml^{-1} or chloramphenicol at 20 µg ml^{-1} prior to connection to the vessel.

2.14 Statistical analysis of results

All statistical tests were carried out using the *t*-table, which allows analysis of normal or approximately normal distributions of data. Therefore it was important to ensure that the results were from normal distributions before 95% confidence limits or student *t*-tests could be used for analysis. Rankits were used to determine the normality of distribution (Wardlaw, 1989). An example of the test is shown in Fig. 2.12.

If the experimental observations are a random sample from an underlying normal distribution, then the observed rankit line (solid) should zigzag in a random fashion fairly closely around the fitted theoretical rankit line (broken) (Wardlaw, 1989). This can be clearly seen in Fig. 2.12 indicating that the experimental results have indeed come from a normal distribution. As a result, *t*-table based statistics are valid. Rankits were used on all sets of data, and all were found to show normal distribution.

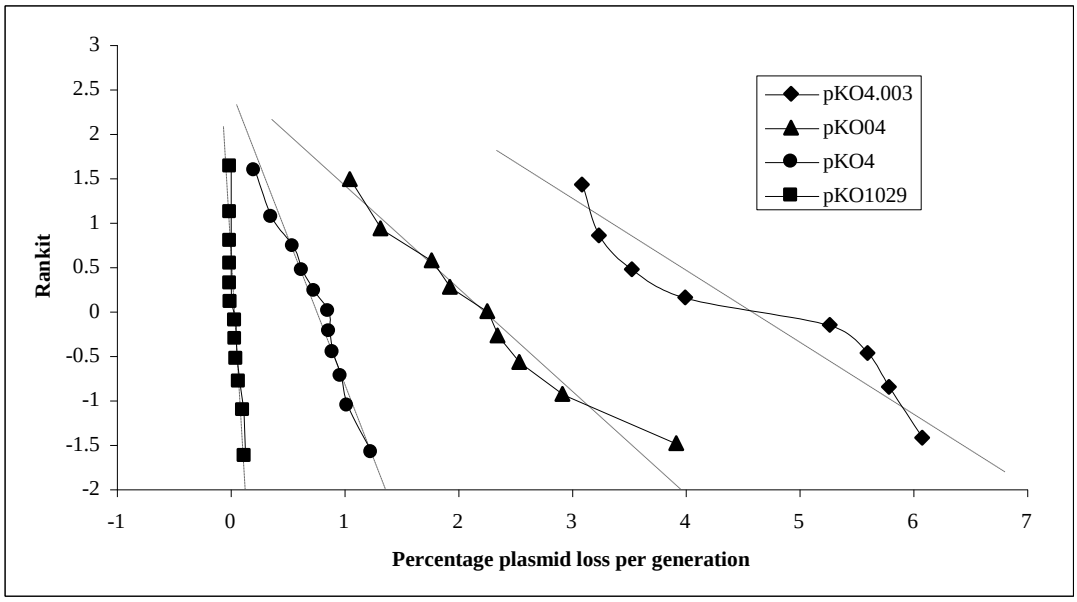


Fig. 2.12: A rankit plot of the percentage plasmid loss per generation for the plasmids pKO1029, pKO4, pKO04 and pKO4.003

2.14.1 95% confidence limits

95% confidence limits (95% CL) were determined on all replicate data produced during this study and used to produce error bars on all graphs and bar charts seen in this thesis.

95% CL = $$\bar{x} \pm t_{(0.05)}\left(\frac{s}{\sqrt{n}}\right)$$

Where $$\bar{x} = \frac{\sum x}{n}$$

$t_{(0.05)}$ is the *t*-value for *n*-1 degrees of freedom,

n is the number of replicates in the distribution,

and $$s = \sqrt{\frac{\sum x^2 - \frac{\left(\sum x\right)^2}{n}}{n-1}}$$ is the standard deviation.

2.14.2 Student *t*-test

The Student *t*-test was used to accept or reject the null hypothesis 'the two means being compared come from a single underlying distribution and differ from each other by no more than can reasonably be explained by random sampling fluctuations' (Wardlaw, 1989).

The 'found value' of *t* when the numbers of replicates of the two experiments are the same was determined as described below.

$$t = \frac{\left(\bar{x}_1 - \bar{x}_2\right)\sqrt{n}}{\sqrt{\left(s_1^2 + s_2^2\right)}}$$ where *s* is the standard deviation (section 2.14.1)

The 'found value' of *t* when the numbers of replicates of the two experiments are different was determined as described below.

$$s = \sqrt{\frac{\left(\sum x_1^2 - \frac{\left(\sum x_1\right)^2}{n_1}\right) + \left(\sum x_2^2 - \frac{\left(\sum x_2\right)^2}{n_2}\right)}{\left(n_1 + n_2\right) - 2}}$$

$$t = \frac{\left(x_1 - x_2\right)}{s\sqrt{\left(\frac{1}{n} + \frac{1}{n_2}\right)}}$$ where *s* is calculated from the formula above

CHAPTER THREE – CONSTRUCTION AND VERIFICATION OF PLASMIDS pKO1029, pKO4, pKO04 AND pKO4.003

3.1 Introduction

The aim of this study was to determine whether decreasing the stability of an antibiotic-resistance plasmid would improve the treatment of bacterial infections by restoring sensitivity to antibiotics. Williams *et al*. (1998) carried out stability experiments on four plasmids which conferred resistance to ampicillin and which had different levels of stability, and their work showed that a decrease in plasmid stability led to an increase in the number of ampicillin-sensitive cells within a population. However, ampicillin is only one of the antibiotics currently used in clinical situations (Stratton, 1996), and it is possible that decreasing the stability of a plasmid that confers resistance to another antibiotic would not lead to a similar increase in antibiotic-sensitive cells. The aim of this section of the work was to construct new plasmids that showed the same stability phenotypes as the plasmids used by Williams *et al*. (1998), but conferred resistance to a different antibiotic. These new plasmids would then be used to determine whether different modes of antibiotic action would affect the apparent stability of the plasmids under experimental conditions.

Ampicillin is a bactericidal antibiotic, and bacterial cells sensitive to ampicillin are killed. As a result, restoring sensitivity to ampicillin will reduce cell numbers within a population in the presence of ampicillin (Livermore & Williams, 1996). Chloramphenicol is a bacteriostatic antibiotic, and does not kill sensitive cells but prevents their replication and cell growth. Therefore, the number of cells within a population will not be reduced by the action of chloramphenicol alone (Stratton, 1996). In order to determine whether the difference in the modes of action of ampicillin and chloramphenicol would affect the results obtained from plasmid stability experiments, the four plasmids used by Williams *et al*. (1998) – namely pALA1029, pOG4, pOG04 and pOG4.003 – were altered to confer resistance to chloramphenicol by inserting the chloramphenicol-resistance gene of transposon Tn*9*.

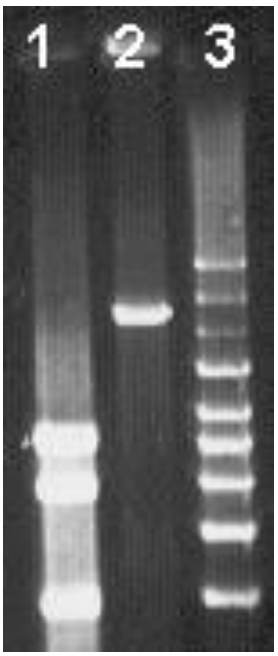
Plasmids pKO1029, pKO4 and pKO4.003 were constructed prior to this study (Oliver, unpublished), although their structures were only verified during this study. Plasmid

pKO04 was constructed and verified during this study. As all these plasmids were constructed using the same method, discussions will relate to all four. The structures of the four plasmids are shown diagrammatically in Figs. 2.7 - 2.10.

3.2 Construction of plasmids that confer resistance to chloramphenicol

Plasmid pJL3-1974 (Fig. 2.6) was used as the source of the chloramphenicol resistance gene, *cat*, which produces chloramphenicol acetyl transferase (Machida *et al*., 1983; Ishizaki & Ohtsubo, 1984). This plasmid carries transposon Tn*9*, which contains *cat* between its flanking IS*1* insertion sequences (Alton & Vapnek, 1979). Each flanking IS*1* insertion sequence contains a single *Pst*I site (Ohtsubo & Ohtsubo, 1978). Plasmids pALA1029, pOG4, pOG04 and pOG4.003 all contain a single *Pst*I site in the centre of the ampicillin resistance gene (Bolivar *et al*., 1977).

Plasmids pALA1029, pOG4, pOG04, pOG4.003 and pJL3-1974 were digested with *Pst*I (section 2.6) and agarose gel electrophoresis (section 2.7) was carried out. Fig. 3.1 shows the *Pst*I digests of plasmids pOG04 and pJL3-1974 after agarose gel electrophoresis.



Lane 1 Plasmid pJL3-1974 digested with *Pst*I (approximately 2.4 kb, 1.9 kb, 1.0 kb).

Lane 2 Plasmid pOG04 digested with *Pst*I (approximately 6.1 kb).

Lane 3 DNA size marker ladder (10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb and 1 kb).

Fig. 3.1: Plasmids pJL3-1974 and pOG04 digested with *Pst*I

Plasmid pJL3-1974 is 5.3 kb in size (Machida *et al*., 1983; Ishizaki & Ohtsubo, 1984). Of the three bands produced by *Pst*I digestion shown in Fig. 3.1 (lane 1), the middle-sized 1.9 kb band contains the chloramphenicol resistance gene (Alton & Vapnek, 1979). Comparison of the middle sized band to that of the DNA marker ladder shows that the band is the expected size.

Plasmid pOG04 is approximately 6.1 kb in size (Macartney *et al*., 1997) and contains only one *Pst*I site. Lane 2 in Fig. 3.1 shows that complete digestion of the plasmid has taken place. Comparison of the single plasmid band with the size marker ladder suggests the plasmid is slightly larger than expected; however, this effect is probably due to the inability of a 0.8% agarose gel to accurately separate large DNA molecules.

The 1.9 kb fragment of pJL3-1974 was to be ligated into each of the digested plasmids, but ligation cannot be carried out in the presence of agarose gel. Therefore the DNA was cut from the agarose gel and purified (section 2.10). Following purification, the pJL3-1974 fragment DNA was mixed with the digested plasmid DNA and ligation was carried out (section 2.11).

Following ligation, the resulting DNA was transformed (section 2.9) into competent *E. coli* DH5α cells (section 2.8). The transformed cells were plated onto an LB agar plate (section 2.3.1) containing chloramphenicol at a concentration of 10 μg ml^{-1} and incubated overnight at 37°C. This selected for cells containing a functional plasmid that conferred chloramphenicol resistance. Each colony was transferred into a flask containing 25 ml of LB broth (section 2.3.1) containing chloramphenicol at a concentration of 20 μg ml^{-1} for overnight growth at 37°C. The plasmids were extracted (section 2.5) to produce stocks of the ligated plasmids.

3.3 Verification of the new ligated plasmids by digestion with *Pst*I

The ligated plasmids all bestowed chloramphenicol resistance on the host bacteria, and therefore were functional to that extent. However, it was important to determine that the fragment of DNA from pJL3-1974 and the original plasmid DNA were both present and of the correct size. All extracted plasmids were therefore digested with *Pst*1

(section 2.6) and gel electrophoresis was carried out (section 2.7). The agarose gels are shown in Figs. 3.2 and 3.3.

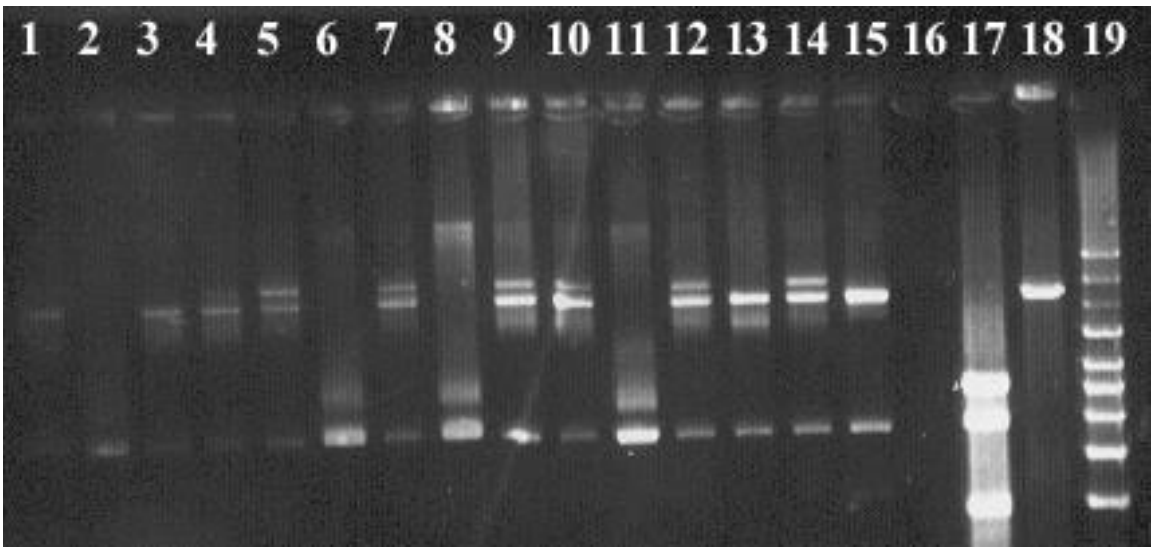


Fig. 3.2: Plasmids created from ligation between pOG04 and the 1.87 kb fragment of pJL3-1974 digested with *Pst*I

Lanes 1-15	Plasmids numbered 1 to 15 produced from ligation of pOG04 and the 1.9 kb fragment of pJL3-1974 digested with *Pst*I
Lane 16	Blank
Lane 17	*Pst*I digested pJL3-1974 (approximately 2.4 kb, 1.9 kb, 1.0 kb)
Lane 18	*Pst*I digested pOG04 (approximately 6.1 kb)
Lane 19	DNA size marker ladder (10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb and 1 kb).

Lane 17 in Fig. 3.2 shows the three fragments produced from digestion of pJL3-1974 with *Pst*I, the middle band of approximately 1.9 kb containing the chloramphenicol resistance gene, and lane 18 shows a single band of *Pst*I-digested pOG04 of approximately 6.1 kb. Lanes 2, 6, 8 and 11 show an unusual pattern of digestion; the plasmids do not have the right combination of fragments. Lane 1 cannot be clearly visualised due to a low quantity of DNA. Lanes 3, 4, 5, 7, 9, 10, 12, 13, 14 and 15 all show partial digestion of the plasmid DNA, and some chromosomal contamination. However, all these lanes contain the 6.1kb band of pOG04 and the 1.9 kb fragment from pJL3-1974. This shows that the correct fragments are present in these 11 ligated plasmids.

In Fig. 3.3, lane 19 shows the three fragments produced from digestion of pJL3-1974 with *Pst*I, the middle band of approximately 1.9 kb containing the chloramphenicol resistance gene. Lane 5 shows a single band of *Pst*I digested pOG4 of approximately 12.7 kb. Lanes 1 to 4 contain this band and the 1.9 kb band of pJL3-1974, showing the correct fragments are present in these 4 ligated plasmids. Lane 11 shows a single band of *Pst*I digested pALA1029 of approximately 8.8 kb. Lanes 7 to 10 contain this band and the 1.9 kb band of pJL3-1974, showing the correct fragments are present in these 4 ligated plasmids. Lane 17 shows a single band of *Pst*I digested pOG4.003 of approximately 12.7 kb. Lanes 13 to 16 contain this band and the 1.9 kb band of pJL3-1974, showing the correct fragments are present in these 4 ligated plasmids.

3.4 Verification of the new ligated plasmids by digestion with *Eco*RI

All the ligated plasmids were digested with *Eco*RI to allow determination of the orientation of the insert and confirmation that only one copy of the insert was present in the plasmids, and also another check on the structural validity. This was carried out in parallel to the *Pst*I digestion.

During the ligation procedure it was not possible to control the direction of insertion of the fragment. The fragment could have inserted into the original plasmids in one of two ways, either with the reading frame of the chloramphenicol resistance gene running clockwise, or running anticlockwise. It was not known whether the direction of transcription would have any effect on stability; however, it was considered beneficial to ensure that the direction of insertion was the same in all the newly constructed plasmids.

The fragment of pJL3-1974 containing the chloramphenicol resistance gene contains a single *Eco*RI site towards the beginning of the reading frame, while the original plasmids also contain a single *Eco*RI site. Digestion of the ligated plasmids would therefore produce two fragments. The size of the larger fragment would vary depending on the size of the original plasmid; the size of the smaller fragment would vary depending on the orientation of the insert. Figs. 3.4 and 3.5 show the two possible configurations of the plasmid ligated from pOG04 and the 1.9 kb fragment of pJL3-1974. For a key to the abbreviations used in the Figs., refer to appendix A.

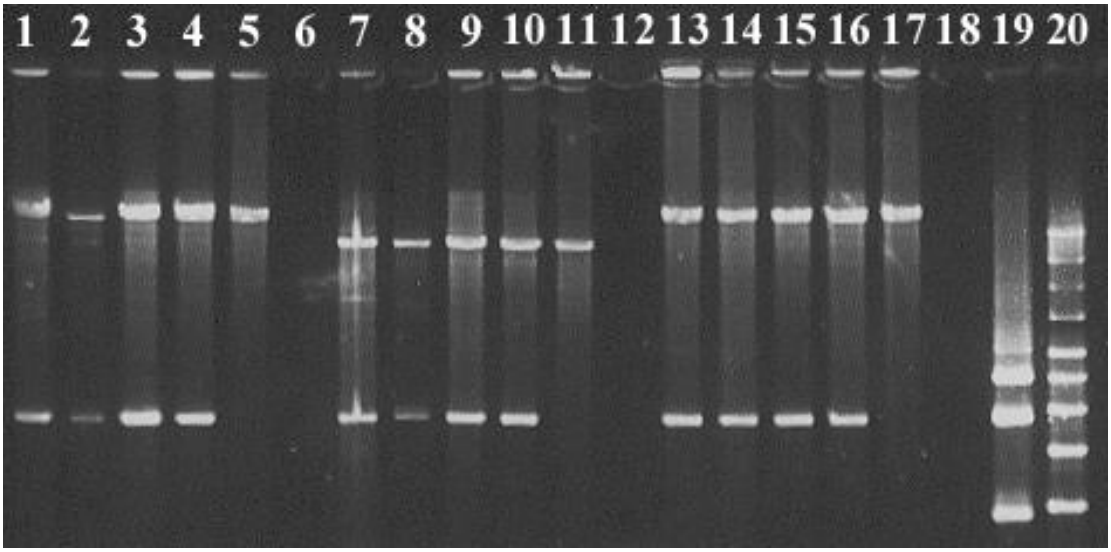


Fig. 3.3: Plasmids created from ligation between pOG4, pALA1029, pOG4.03 and the fragment of pJL3-1974 digested with *Pst*I

Lanes 1-4	Plasmids numbered 1 to 4 produced from ligation of pOG4 and the 1.9 kb fragment of pJL3-1974 digested with *Pst*I
Lane 5	*Pst*I digested pOG4 (approximately 12.7 kb)
Lane 6	Blank
Lanes 7-10	Plasmids numbered 1 to 4 produced from ligation of pALA1029 and the 1.9 kb fragment of pJL3-1974 digested with *Pst*I
Lane 11	*Pst*I digested pALA1029 (approximately 8.8 kb)
Lane 12	Blank
Lanes 13-16	Plasmids numbered 1 to 4 produced from ligation of pOG4.003 and the 1.9 kb fragment of pJL3-1974 digested with *Pst*I
Lane 17	*Pst*I digested pOG4.003 (approximately 12.7 kb)
Lane 18	Blank
Lane 19	*Pst*I digested pJL3-1974 (approximately 2.4 kb, 1.9 kb, 1.0 kb)
Lane 20	DNA size marker ladder (10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb and 1 kb).

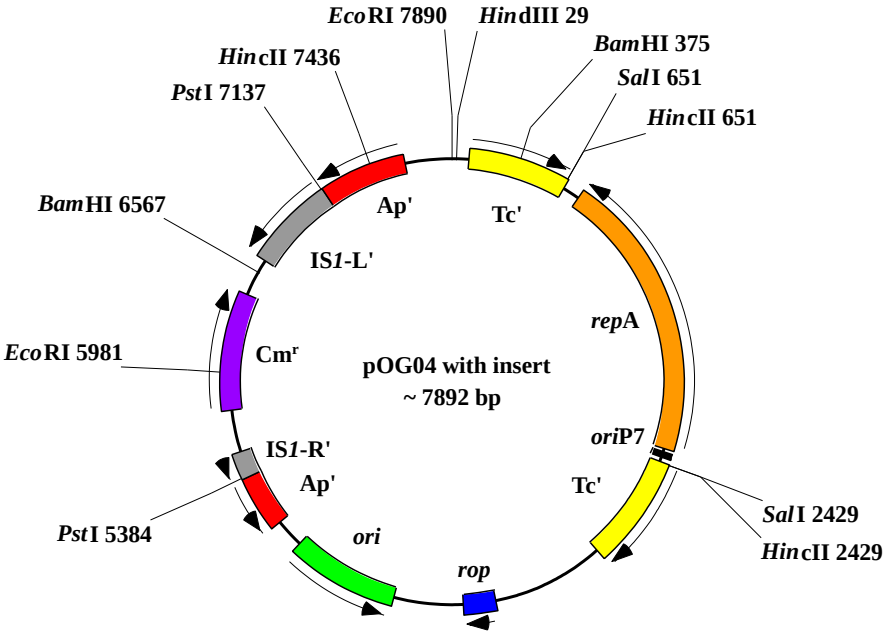


Fig. 3.4: **The structure of the plasmid produced from a ligation between pOG04 and the fragment of pJL3-1974 when the reading frame of the chloramphenicol resistance gene runs clockwise.**

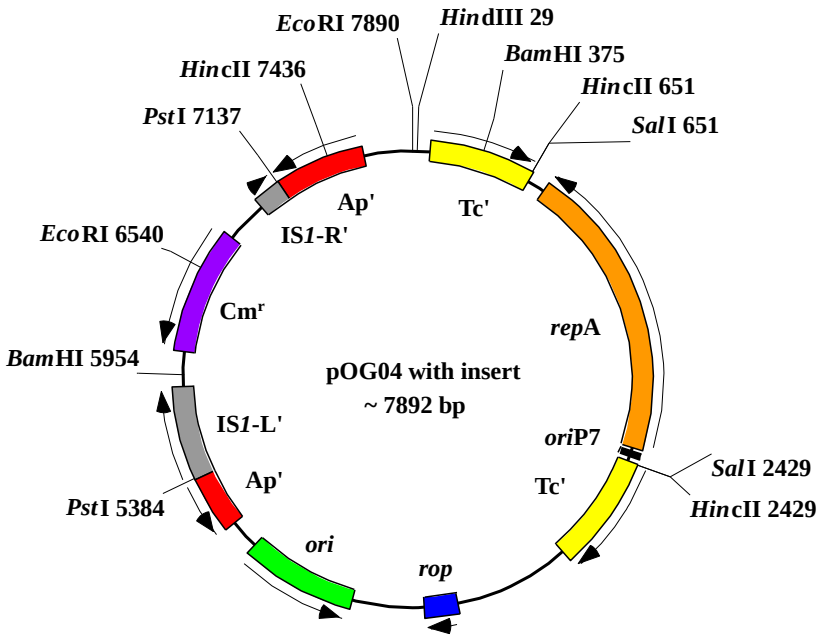


Fig. 3.5: **The structure of the plasmid produced from a ligation between pOG04 and the fragment of pJL3-1974 when the reading frame of the chloramphenicol resistance gene runs anticlockwise.**

If the chloramphenicol resistance gene were inserted with the reading frame running clockwise (in the opposite direction to that of the ampicillin resistance gene), a digest with *Eco*RI would produce two fragments, the smallest being approximately 1.9 kb. However, if the chloramphenicol resistance gene were inserted with the reading frame running anticlockwise, the smallest fragment would be approximately 1.4 kb. The difference in size between these two fragments would be clearly visible when run on a 0.8% agarose gel.

All the ligated plasmids were digested with *Eco*RI (section 2.6) and agarose gel electrophoresis was carried out (section 2.7). The agarose gels are shown in Figs. 3.6 and 3.7.

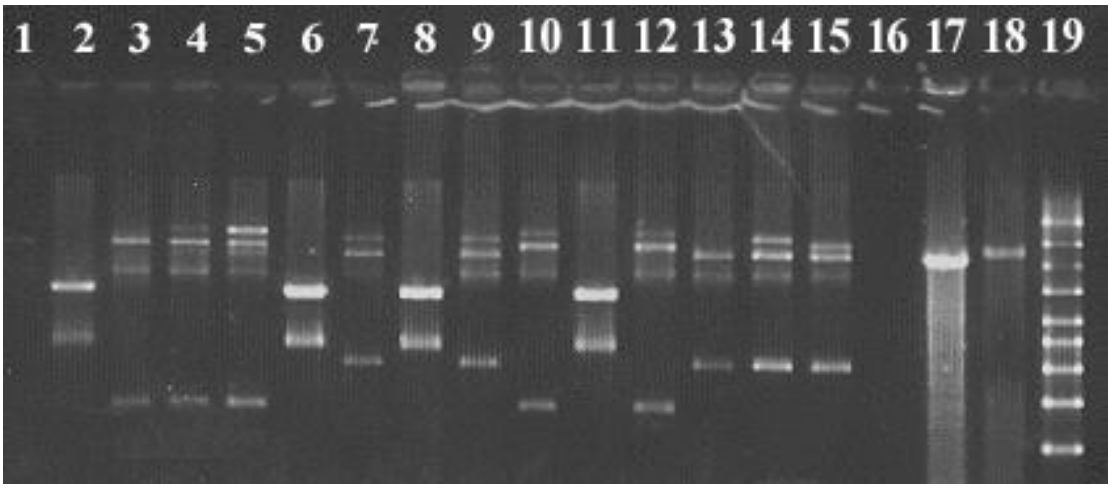


Fig. 3.6: Plasmids created from ligation between pOG04 and the fragment of pJL3-1974 digested with *Eco*RI

Lanes 1-15	Plasmids numbered 1 to 15 produced from ligation of pOG04 and the 1.9 kb fragment of pJL3-1974 digested with *Eco*RI
Lane 16	Blank
Lane 17	*Eco*RI digested pJL3-1974 (5.3 kb)
Lane 18	*Eco*RI digested pOG04 (approximately 6.1 kb)
Lane 19	DNA size marker ladder (10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb and 1 kb).

Fig. 3.6 verifies the presence of a single *Eco*RI site in both pJL3-1974 (lane 17) and pOG04 (lane 18). The digest in lane 1 could again not be visualised due to a small

quantity of DNA, and the unusual structure of the plasmids numbered 2, 6, 8 and 11 is confirmed. As a result these plasmids were discarded. Partial digestion and chromosomal contamination can also be seen in the plasmid digests. The plasmids run in lanes 3, 4, 5, 10 and 12 contain the chloramphenicol resistance gene with the reading frame running in an anticlockwise direction shown by the presence of the smaller *Eco*RI fragment. The plasmids run in lanes 7, 9, 13, 14 and 15 contain the chloramphenicol resistance gene with the reading frame running in a clockwise direction shown by the presence of the larger fragment. Half of the plasmid stock contains the insert in an anticlockwise orientation; the other half contains the insert in a clockwise orientation, suggesting that the orientation of the fragment has no effect on the plasmid, either as an enhancement or detriment to its function.

In Fig. 3.7, lane 18 confirms that pJL3-1974 contains a single *Eco*RI site. Lane 5 confirms that pOG4 also contains a single *Eco*RI site, lane 1 shows a ligated plasmid where the insert is present anticlockwise, and lanes 2 and 4 show ligated plasmids where the insert is present clockwise. The plasmid present in lane 3 contains two inserts, both running in tandem in a clockwise direction. Lane 11 confirms that pALA1029 contains a single *Eco*RI site, lanes 7, 8 and 10 show ligated plasmids where the insert is present anticlockwise, and lane 9 shows a ligated plasmid with the insert present clockwise. Lane 16 confirms that pOG4.003 contains a single *Eco*RI site, lanes 12, 13 and 15 contain ligated plasmids where the insert is present clockwise, and lane 14 shows a ligated plasmid with the insert present in an anticlockwise direction.

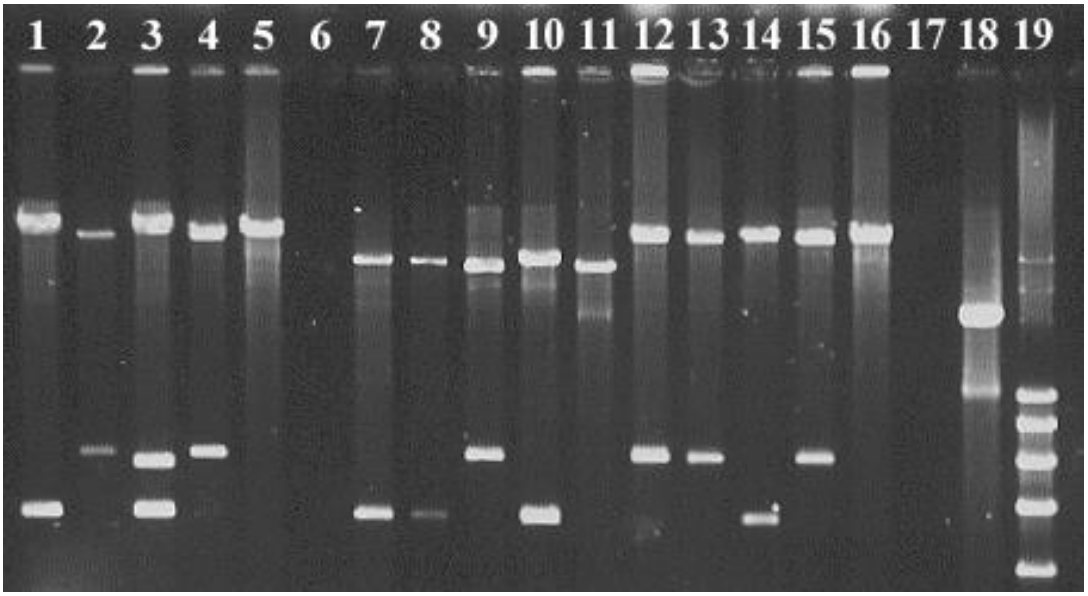


Fig. 3.7: Plasmids created from ligation between pOG4, pALA1029, pOG4.03 and the fragment of pJL3-1974 digested with *Eco*RI

Lane 1-4	Plasmids numbered 1 to 4 produced from ligation of pOG4 and the 1.9 kb fragment of pJL3-1974 digested with *Eco*RI
Lane 5	*Eco*RI digested pOG4 (approximately 12.7 kb)
Lane 6	Blank
Lane 7-10	Plasmids numbered 1 to 4 produced from ligation of pALA1029 and the 1.9 kb fragment of pJL3-1974 digested with *Eco*RI
Lane 11	*Eco*RI digested pALA1029 (approximately 8.8 kb)
Lane 12-15	Plasmids numbered 1 to 4 produced from ligation of pOG4.003 and the 1.9 kb fragment of pJL3-1974 digested with *Eco*RI
Lane 16	*Eco*RI digested pOG4.003 (approximately 12.7 kb)
Lane 17	Blank
Lane 18	*Eco*RI digested pJL3-1974 (5.3 kb)
Lane 19	DNA size marker ladder (10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb and 1 kb).

3.5 Selection of the plasmids pKO1029, pKO4, pKO04 and pKO4.003 from the newly ligated plasmid stocks

As a result of the agarose gels shown in Figs. 3.2, 3.3, 3.6 and 3.7, the following four plasmids were chosen for use in stability experiments:

pKO1029 Plasmid number 3 produced from the ligation of pALA1029 and the 1.9 kb fragment of pJL3-1974 (approximately 10.5 kb),

pKO4 Plasmid number 4 produced from the ligation of pOG4 and the 1.9 kb fragment of pJL3-1974 (approximately 14.5 kb),

pKO04 Plasmid number 15 produced from the ligation of pOG04 and the 1.9 kb fragment of pJL3-1974 (approximately 7.9 kb),

pKO4.003 Plasmid number 1 produced from the ligation of pOG4.003 and the 1.9 kb fragment of pJL3-1974 (approximately 14.5 kb).

All these plasmids contain the chloramphenicol resistance gene running in an anticlockwise orientation and appear structurally correct. The other plasmids were stored.

3.6 Estimation of relative plasmid copy number for the plasmids pKO1029, pKO4, pKO04 and pKO4.003

Plasmid copy number can have a dramatic effect on plasmid stability (Leonhardt & Alonso, 1988; Allen & Blaschek, 1990). The plasmid copy number is the number of plasmid copies present per cell on average throughout the whole population. Techniques for determining plasmid copy number are useful for analysing the effect of plasmid burden on a host cell, but frequently the techniques are time-consuming or costly (Schmidt *et al*., 1996).

Williams *et al*. (1998) determined the relative plasmid copy number of plasmids pALA1029, pOG4, pOG04 and pOG4.003 in order to assist with the interpretation of the stability experiments. The relative plasmid copy number is the average number of plasmid copies per chromosome within a population. Determination of relative plasmid copy number is relatively simple, and yet still provides some indication of the level of plasmid burden on a host cell.

The determination of relative plasmid copy number for the plasmids pKO1029, pKO4, pKO04 and pKO4.003 was determined as insertion of the fragment from pJL3-1974 might have caused changes to the relative plasmid copy number, which would in turn affect the stability of the plasmids.

In order to determine relative plasmid copy number, total DNA was extracted from *E. coli* C2110 colonies transformed with each of the plasmids pKO1029, pKO4, pKO04 and pKO4.003 (section 2.12.1). The total DNA extracts were digested with *Eco*RI (section 2.6) and gel electrophoresis (section 2.7) was carried out on these samples. An *Eco*RI digest of plasmid pOG004 (Fig. 2.11) was also run on this gel to provide a control for the probe. Fig. 3.8 shows the appearance of the gel. Southern blotting (section 2.12.3) was carried out on the gel shown in Fig. 3.8 and the blot was radioactively probed (sections 2.12.4 to 2.12.7) with pOG004 DNA. This plasmid (Fig. 2.11) contains sequences that would bind to both chromosomal and plasmid DNA. The blot was exposed to photographic film for one week (section 2.12.8) and the autoradiograph produced from this procedure is shown in Fig. 3.9.

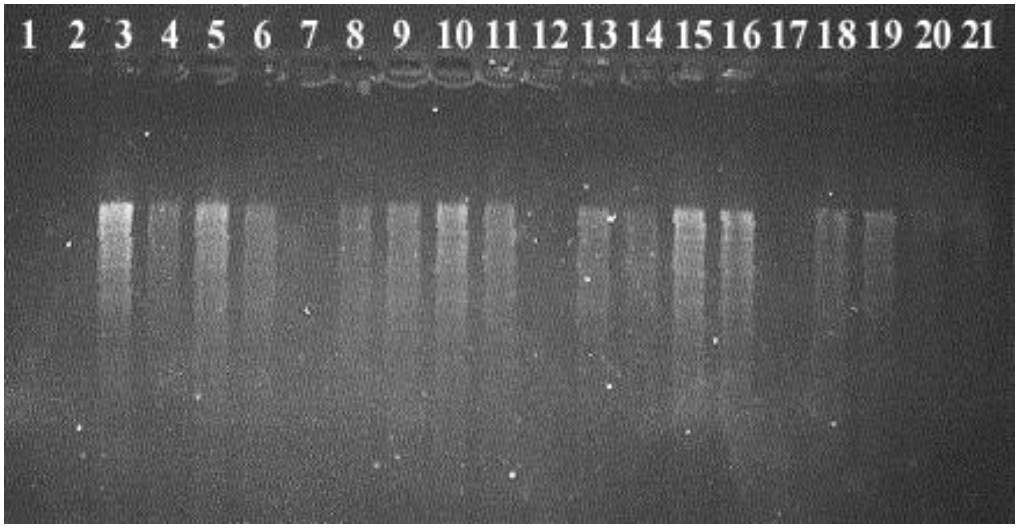


Fig. 3.8: Total DNA extracted from E. coli C2110 transformed with pKO04, pKO4, pKO4.003 or pKO1029 and digested with *Eco*RI, and pOG004 digested with *Eco*RI

Lane 1	Plasmid pOG004 digested with *Eco*RI
Lane 2	Blank
Lanes 3-6	Total DNA extracted from *E. coli* C2110 transformed with pKO04 and digested with *Eco*RI
Lane 7	Blank
Lanes 8-11	Total DNA extracted from *E. coli* C2110 transformed with pKO4 and digested with *Eco*RI
Lane 12	Blank
Lanes 13-16	Total DNA extracted from *E. coli* C2110 transformed with pKO4.003 and digested with *Eco*RI
Lane 17	Blank
Lanes 18-21	Total DNA extracted from *E. coli* C2110 transformed with pKO1029 and digested with *Eco*RI

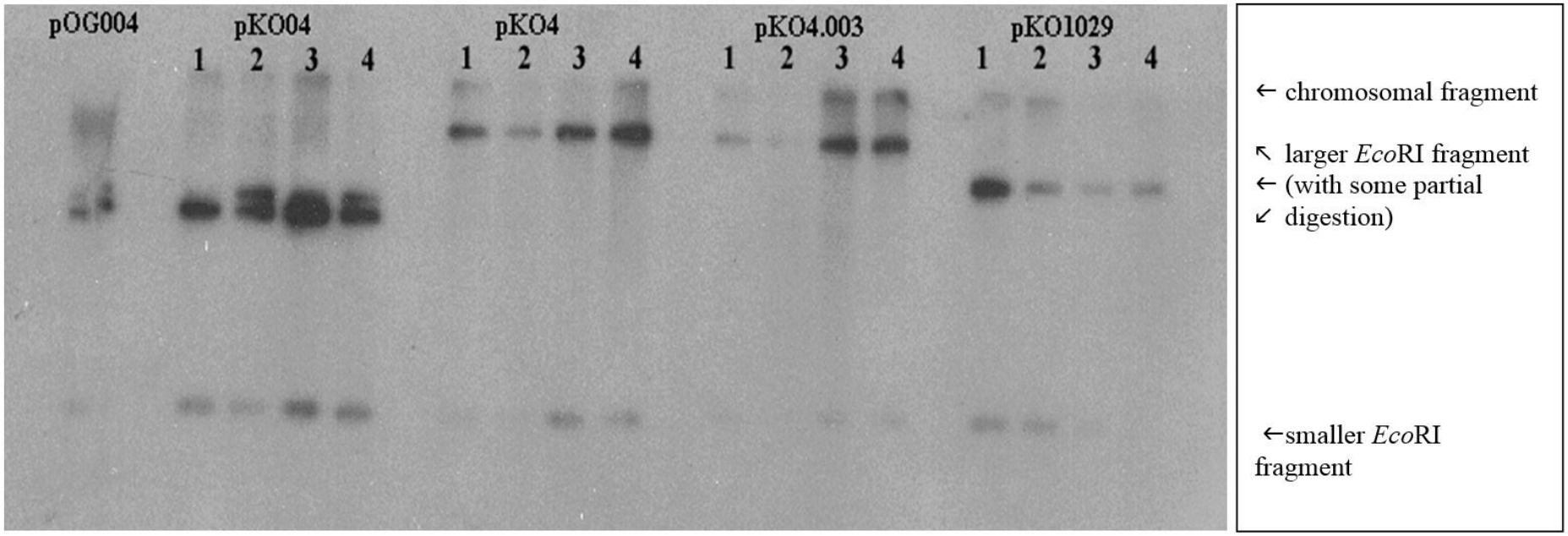


Fig. 3.9: **Autoradiograph showing bands of chromosomal and plasmid DNA produced by binding of a radioactively labelled pOG004 probe.**

The top bands in Fig. 3.9 are the chromosomal fragments containing the *trp*E gene homologous to that present in plasmid pOG004. The lowest bands are the small *Eco*RI fragments produced from plasmid digestion. The middle bands are the larger *Eco*RI fragments from the plasmids. The difference in size of the plasmids causes the variation in position of the middle fragments on the blot (see section 3.4). Partial digestion of the plasmids has occurred, particularly in the total DNA extracts from *E. coli* C2110 containing pKO04. The band for plasmid pOG004 was disrupted due to a split in the gel.

In order to determine the relative copy number of a plasmid, the intensity of the chromosomal band is compared to that of the plasmid bands. If the plasmid bands are twice as dark as the chromosomal band, then the plasmid has a relative copy number of two. However, photographic film does not become darker on exposure to radioactivity in a linear manner. As a result, the blot obtained in this study cannot be used to precisely enumerate the relative copy number of the plasmid to the chromosome. Nevertheless, the blot can be compared to those produced for the Williams *et al*. (1998) paper. In this paper the blots were quantified by use of a phosphoimager, which does show a linear increase in band intensity on exposure to radioactivity. Fig. 3.10 shows the phosphorimager image obtained for the 1998 paper.

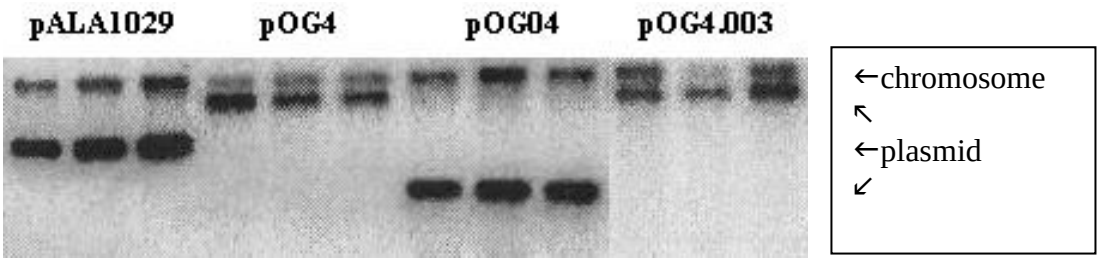


Fig. 3.10: Phosphorimager image showing bands of chromosomal and plasmid DNA produced by binding of a radioactively labelled pOG004 probe.

From the phosphoimager results shown in Fig. 3.10, the pALA1029 band was seen to be almost five times darker than the chromosomal band; the relative plasmid copy number was calculated to be 4.96 ± 2.58. The other plasmid bands were all approximately twice as dark as the chromosomal bands; the relative plasmid copy number for pOG4 was 1.95 ± 1.54, for pOG04 the relative copy number was 2.09 ±

0.19, and for pOG4.003 the relative copy number was 1.88 ± 0.37 (Williams *et al*., 1998).

Fig. 3.9 shows that the larger plasmid fragment of pKO1029 is significantly darker than the chromosomal band, as was seen for pALA1029 in Fig. 3.10. This is particularly noticeable in extract 1, suggesting that this plasmid still shows a high relative plasmid copy number.

The difference between the intensity of the chromosomal band and the pKO4 plasmid band was less than that seen for pKO1029, suggesting that the relative plasmid copy number for this plasmid is lower than that of pKO1029. Equally, the difference in intensity between bands from total DNA extracts of *E. coli* C2110 containing plasmids pKO4.003 was less than that seen for pKO1029, again suggesting that the relative plasmid copy number was lower than that of pKO1029. In fact, the band intensities for pKO4 extract 4 and pKO4.003 extracts 3 and 4 are very similar, suggesting these plasmids show approximately the same relative plasmid copy number.

The plasmid bands from extracts of *E. coli* C2110 containing pKO04 appear much darker than the chromosomal bands, even to the extent of a larger difference in intensity than that seen for pKO1029. This may suggest that the relative copy number has increased slightly for plasmid pKO04, which may cause an increase in plasmid stability.

3.7 Conclusion

The plasmids pKO1029, pKO4, pKO04 and pKO4.003 all conferred resistance to chloramphenicol on the host cells *E. coli* C2110 and *E. coli* DH5α, were structurally sound as far as could be determined, and contained a single insert in a known orientation. The only difference between plasmids pKO1029, pKO4, pKO04, and pKO4.003 and plasmids pALA1019, pOG4, pOG04, and pOG4.003 was the ability to confer resistance to chloramphenicol instead of resistance to ampicillin. Plasmid pKO04 may have an increased relative copy number, the effect of which could perhaps be evident in further experiments. The stability of these eight plasmids in batch culture was next determined, by the technique used by Williams *et al*. (1998). As the new

plasmids conferred resistance to chloramphenicol, it was expected that the results would allow comparison of their stability with that of ampicillin resistance plasmids.

CHAPTER FOUR – MEASUREMENT OF THE STABILITY OF PLASMIDS pALA1029, pOG4, pOG04, pOG4.003, pKO1029, pKO4, pKO04 AND pKO4.003 IN BATCH CULTURE

4.1 Introduction

Accurate measurement of plasmid stability is essential for the study of partitioning systems, as it enables determination of the effect on plasmid stability of the removal of genes involved in plasmid partition (Gallie & Kado, 1987; Macartney *et al*., 1997). It can also be employed to optimise large-scale fermentation processes by identifying the conditions that lead to a decrease in plasmid stability and altering the system to avoid such conditions (Karbasi & Keshavarz, 1997).

Plasmid stability has been measured in the same way for many years. The technique involves batch culture of the organisms in non-selective media, which allows plasmid-free cells to replicate freely. Samples are taken from the cultures at various points, spread plated to obtain a viable count, and then replica plated onto antibiotic-containing media in order to determine the percentage of plasmid-free cells within the sample (Kim & Blaschek, 1989; Roberts & Helinski, 1992; Easter *et al.*, 1998). Sometimes serial dilutions of the culture are made, and these extend the experimental time so that data can be obtained over many generations of cell growth (Simpson *et al*., 2003; Venkova-Canova *et al*., 2003). The results from such experiments are generally expressed as a percentage plasmid loss per generation (Roberts & Helinski, 1992; Easter *et al*., 1997; Macartney *et al*., 1997). Batch cultures of microorganisms are an efficient way to obtain plasmid stability data, as experiments are generally short and therefore mutations that could affect stability will be unlikely to occur over the course of the experiment.

Williams *et al.* (1998) used batch culture techniques to determine the stability of several plasmids including pALA1029, pOG4, pOG04 and pOG4.003. After calculation of the percentage plasmid loss per generation, they chose zones of stability to group apparently similar phenotypes. These zones were designated as ‘stabilised partition’, ‘random partition’ and ‘destabilised partition’. Fig. 4.1 shows these stability groupings as published in Williams *et al*. (1998).

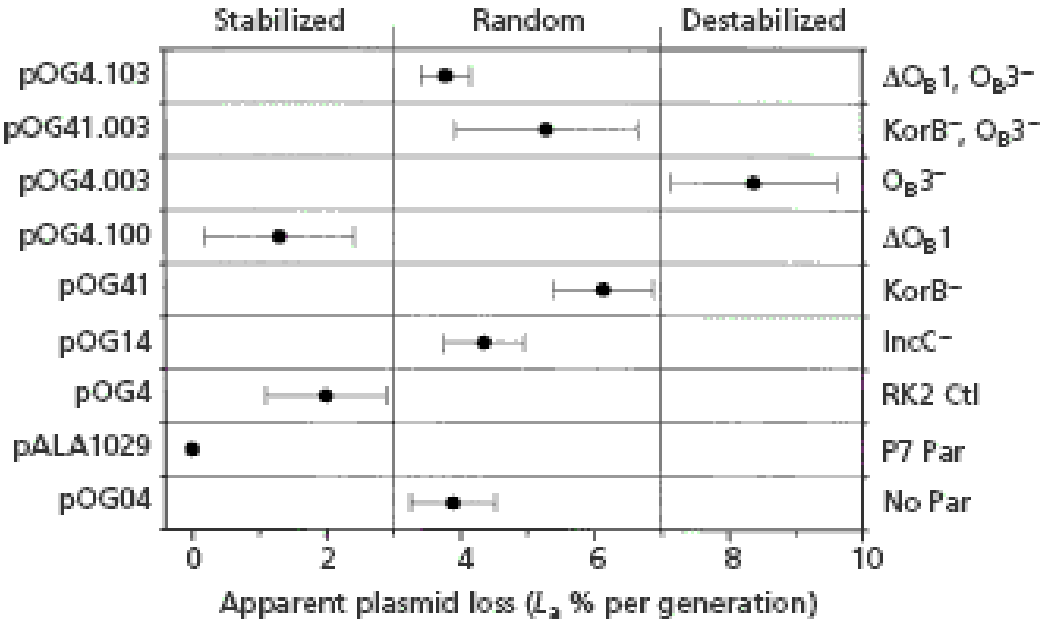


Fig. 4.1: **A chart showing the apparent plasmid loss for a range of plasmids analysed by Williams *et al*. (1998). This shows the plasmids grouped into stabilised, random and destabilised levels of inheritance**

The zones of stability; stabilized, random and destabilized; shown in Fig. 4.1 were chosen as a result of the experiments carried out on the different plasmids. It is clear from the Fig. 4.1 that plasmid pALA1029 is a very stable plasmid showing no plasmid loss over the course of the experiments. Plasmid pOG4 was also described as a stable plasmid as the apparent plasmid loss was relatively low, but also because it was known to contain a functioning partition system (the *ctl* region from plasmid RK2). These plasmids were therefore placed into the stabilized zone. Plasmid pOG04 was shown to be slightly less stable than plasmid pOG4 and pALA1029, and it was also known that it did not contain a partition system. As a result this plasmid was described as having random partition, the situation that would occur if no stability system were present. Many of the other plasmids in the study were seen to show random partition, suggesting that the alterations made to these plasmids had the effect of destroying any partitioning function that may have been present. The unusual result obtained from the Williams *et al*. (1998) study was the result for plasmid pOG4.003, a plasmid in which the O_B3 site has been rendered non-functional. This plasmid showed a higher apparent plasmid loss per generation as compared to plasmids that did not contain a stability system (the

random group). The stability system in this plasmid was having a detrimental affect on plasmid inheritance, and as a result the plasmid was termed destabilized.

The plasmids pALA1029, pOG4, pOG04 and pOG4.003 were chosen for use in the present study because between them they represent each stability phenotype identified in Williams *et al*. (1998) and they therefore provide a good range for determining the effects of a change in plasmid stability on the overall antibiotic resistance of a bacterial cell population.

The aim of this section of work was to replicate some of the experiments published in Williams *et al.* (1998) in order to confirm the stabilities of the plasmids pALA1029, pOG4, pOG04 and pOG4.003, and to carry out a parallel study relating to the stabilities of the newly-constructed plasmids pKO1029, pKO4, pKO04 and pKO4.003.

4.2 Method

As the stability of the plasmids pALA1029, pOG4, pOG04 and pOG4.003 had already been determined (Williams *et al.*, 1998) experiments were carried out not to obtain statistically significant data, but only to ensure that the phenotypes had not altered during the intervening period. Results for the plasmids pALA1029, pOG4 and pOG4.003 were obtained in quadruplicate, and results for the plasmid pOG04 were obtained in triplicate. The results obtained by Williams *et al.* (1998) have been included in Fig. 4.1 and Fig. 4.2 for discussion and comparison with the results obtained in this study.

The stability of the plasmids pKO1029, pKO4, pKO04 and pKO4.003 under batch culture conditions was determined using at least eight separate transformants in order to obtain statistically-significant results.

The batch culture experiments were carried out as described in section 2.13.1, and the apparent loss of plasmids from each experiment was calculated using the formulae given in the same section. The raw data and calculations made from these experiments are shown in Tables 4.1 and 4.2. The mean apparent percentage plasmid loss per generation for each plasmid was determined, and 95% confidence limits were calculated

(section 2.14.1) for use in the bar charts. In order to determine the statistical significance between the results obtained, the Student *t*-test was used (section 2.14.2).

4.3 Results

Table 4.1 shows the results obtained from batch culture experiments carried out on *E. coli* C2110 transformed with the plasmids pALA1029, pOG4, pOG04 and pOG4.003.

Plasmid	CFU's per ml at t_o	CFU's per ml at t_{end}	Number of Generations	Percentage Ap^r at t_o	Percentage Ap^r at t_{end}	Apparent % plasmid loss / gen.
pALA1029	148	1.99×10^9	23.7	98.1	96.8	0.056
	148	2.45×10^9	24.0	100	98.9	0.046
	125	1.90×10^9	23.9	100	100	0.000
	200	3.75×10^9	24.2	100	99.2	0.033
pOG4	345	2.44×10^9	22.8	90.3	33.3	4.28
	286	1.44×10^9	22.3	94.1	69.6	1.34
	109	2.79×10^9	24.6	87.5	51.1	2.16
	81	9.00×10^8	23.4	84.9	70.0	0.82
pOG04	197	1.80×10^9	23.1	78.2	32.3	3.76
	281	3.09×10^9	23.4	84.9	23.5	5.34
	253	3.46×10^9	23.7	83.9	24.5	5.06
pOG4.003	81	2.45×10^9	24.9	68.6	9.34	7.70
	171	2.45×10^9	23.8	79.8	9.48	8.56
	255	2.10×10^9	23.0	79.7	4.18	12.0
	253	1.73×10^9	22.7	76.7	7.13	9.94

Table 4.1: Calculation of the apparent percentage plasmid loss per generation for individual batch culture experiments carried out with *E. coli* C2110 transformed with the plasmids pALA1029, pOG4, pOG04 and pOG4.003

Table 4.2 shows the results obtained from batch culture experiments carried out on *E. coli* C2110 transformed with the plasmids pKO1029, pKO4, pKO04 and pKO4.003.

Plasmid	CFU's per ml at t_o	CFU's per ml at t_{end}	Number of Generations	Percentage Cm^r at t_o	Percentage Cm^r at t_{end}	Apparent % plasmid loss / gen.
pKO1029	254	4.75×10^9	24.2	100	97.0	0.126
	386	5.60×10^9	23.8	100	100	0.000
	328	5.01×10^9	23.9	100	98.7	0.055
	583	5.22×10^9	23.1	99.5	99.4	0.004
	164	3.29×10^9	24.3	98.1	97.1	0.042
	234	1.90×10^9	23.0	100	98.3	0.075
	193	2.18×10^9	23.4	100	100	0.000
	288	2.62×10^9	23.1	100	100	0.000
	130	1.17×10^9	23.1	100	97.4	0.114
	142	1.04×10^9	22.8	100	99.0	0.044
	76	2.18×10^9	24.8	100	100	0.000
	149	3.25×10^9	24.4	100	100	0.000
pKO4	237	5.26×10^9	24.4	99.0	84.8	0.63
	261	4.71×10^9	24.1	98.6	73.0	1.24
	245	1.56×10^9	22.6	98.1	77.6	1.03
	380	5.28×10^9	23.7	98.9	79.8	0.90
	348	2.62×10^9	22.8	95.5	80.7	0.74
	164	2.43×10^9	23.8	97.0	92.2	0.21
	115	2.81×10^9	24.5	93.9	82.0	0.55
	224	2.40×10^9	23.4	93.0	85.5	0.36
	65	1.48×10^9	24.4	90.1	73.0	0.86
	107	2.53×10^9	24.5	85.3	68.8	0.87
	116	1.75×10^9	23.9	98.1	77.7	0.97
pKO04	138	3.23×10^9	24.5	84.8	41.0	2.92
	94	1.38×10^9	23.8	75.5	49.3	1.77
	243	2.28×10^9	23.2	77.9	45.8	2.26
	237	3.48×10^9	23.8	74.7	54.5	1.32
	139	2.17×10^9	23.9	79.5	61.8	1.05
	218	1.12×10^9	22.3	83.5	49.1	2.35
	82	1.70×10^9	24.3	76.9	41.2	2.54
	105	1.05×10^9	23.3	81.0	51.4	1.93
	114	1.24×10^9	23.4	80.3	31.5	3.92
pKO4.003	89	2.28×10^9	24.6	56.2	25.0	3.24
	136	1.62×10^9	23.5	58.1	27.8	3.09
	205	2.46×10^9	23.5	61.5	14.1	6.08
	222	3.10×10^9	23.7	71.2	17.3	5.79
	145	1.33×10^9	23.1	50.0	21.8	3.53
	177	2.04×10^9	23.5	58.9	15.2	5.60
	126	2.38×10^9	24.2	57.5	21.4	4.00
	186	3.32×10^9	24.1	71.2	19.3	5.27

Table 4.2: Calculation of the apparent percentage plasmid loss per generation for individual batch culture experiments carried out with *E. coli* C2110 transformed with the plasmids pKO1029, pKO4, pKO04 and pKO4.003

The mean apparent percentage plasmid loss per generation for plasmids pALA1029, pOG4, pOG04 and pOG4.003, with 95% confidence limits for each plasmid, is shown in Fig. 4.2. Also included in Fig. 4.2 are the mean apparent percentage plasmid loss per generation for plasmids pALA1029, pOG4, pOG04 and pOG4.003 obtained by Williams *et al*. (1998). The mean apparent percentage plasmid loss per generation for plasmids pKO1029, pKO4, pKO04 and pKO4.003, with 95% confidence limits for each plasmid, is shown in Fig. 4.2.

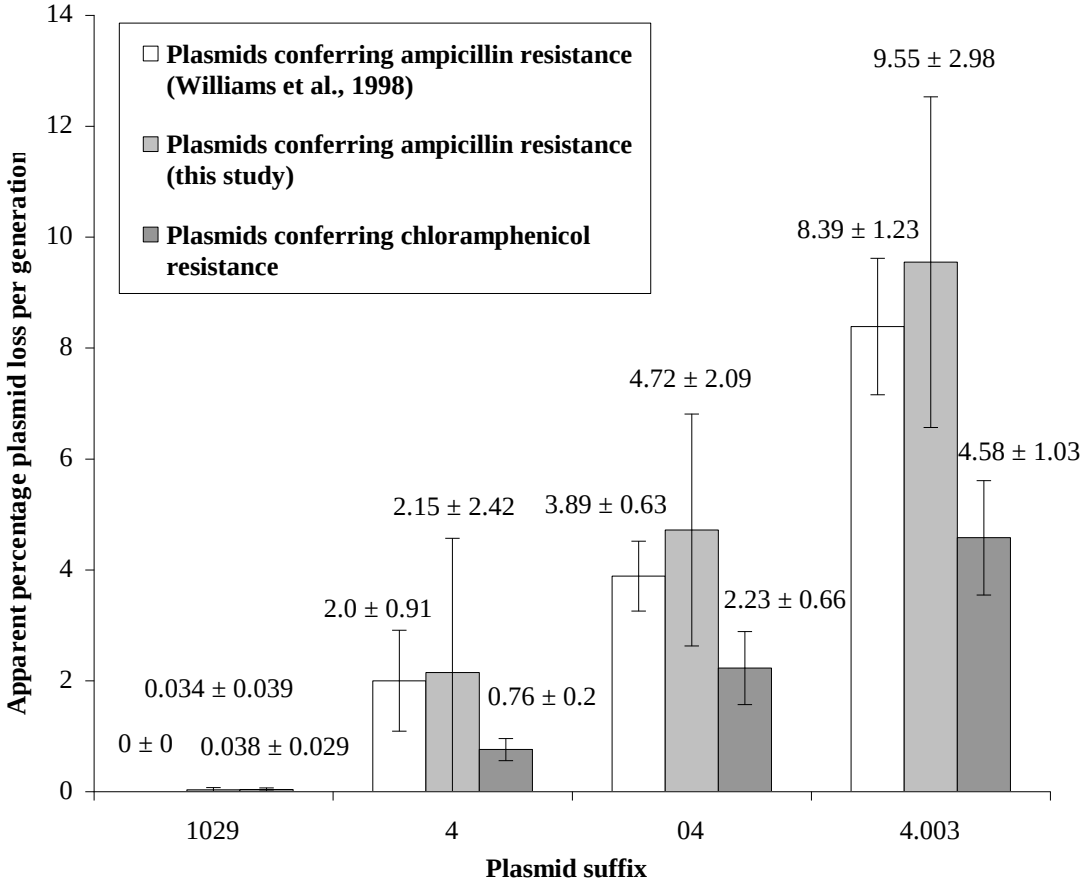


Fig. 4.2: The mean apparent percentage plasmid loss per generation for plasmids pALA1029, pOG4, pOG04 and pOG4.003 obtained from this study and Williams *et al*. (1998), and for plasmids pKO1029, pKO4, pKO04 and pKO4.003, showing 95% confidence limits

It can be seen that cultures containing pALA1029 show the lowest mean apparent percentage plasmid loss per generation of the plasmids conferring ampicillin resistance, indicating that the plasmid is efficiently inherited by daughter cells. The difference

between the result obtained by Williams *et al*. (1998) and the result obtained in this study is highly significant ($P < 0.1\%$). Plasmid pKO1029 shows the most stable inheritance of the plasmids conferring chloramphenicol resistance with cultures having a low mean percentage plasmid loss per generation. There is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid loss per generation for plasmid pALA1029 obtained in this study, and the mean apparent percentage plasmid loss per generation for plasmid pKO1029. There is, however, a significant difference ($1\% > P > 0.5\%$) between the mean apparent percentage plasmid loss per generation of plasmid pALA1029 obtained by Williams *et al*. (1998), and the mean apparent percentage plasmid loss per generation for plasmid pKO1029.

Plasmid pOG4 shows a reasonable degree of stability. The difference between the result obtained in this study and the result obtained by Williams *et al*. (1998) is not significant ($P > 20\%$). Plasmid pKO4 also shows a reasonable degree of stability, where the plasmid is slightly less stable than pKO1029. The mean apparent percentage plasmid loss per generation for pKO4 is significantly different ($1\% > P > 0.5\%$) to that of pOG4 obtained by Williams *et al*. (1998). There is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid loss per generation for pOG4 obtained in this study and that of pKO4.

Cultures containing pOG04 have a high mean apparent percentage plasmid loss per generation. There is no significant difference ($20\% > P > 10\%$) between the results obtained by Williams *et al*. (1998) and the results obtained in this study. Plasmid pKO04 shows a high degree of instability as compared to plasmids pKO1029 and pKO4. There is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid loss per generation for pKO04 and the result for pOG04 obtained in this study. There is a highly significant ($P < 0.1\%$) difference between the result for pOG04 obtained by Williams *et al*. (1998) and pKO04.

Plasmid pOG4.003 shows the highest instability of the four ampicillin-conferring plasmids. The difference between the results obtained by Williams *et al*. (1998) and the results obtained in this study are not significant ($20\% > P > 10\%$). Plasmid pKO4.003 shows the highest instability of the four chloramphenicol-conferring plasmids. The difference between the mean percentage plasmid loss per generation of pKO4.003 and

that of pOG4.003 is highly significant ($P < 0.1\%$) for the results obtained both in this study and by Williams *et al*. (1998).

The difference between the mean apparent percentage plasmid loss per generation for pOG4 and pALA1029 is highly significant ($P < 0.1\%$) for the results obtained for Williams *et al*. (1998), and significant ($5\% > P > 2\%$) for the results obtained in this study. There is a highly significant difference ($0.5\% > P > 0.1\%$) between the mean apparent percentage plasmid loss per generation of pOG04 and pOG4 from the results obtained by Williams *et al*. (1998); there is a significant ($5\% > P > 2\%$) difference between the results obtained in this study. The difference between the mean apparent percentage plasmid loss per generation of pOG4.003 and pOG04 was highly significant ($P < 0.1\%$) according to the results published in Williams *et al*. (1998), and significant ($1\% > P > 0.5\%$) according to the results obtained in this study.

There is a highly significant ($P < 0.1\%$) difference between the mean apparent percentage plasmid loss per generation of pKO1029 and pKO4. Plasmid pKO04 is inherited less effectively than pKO4; there is a highly significant ($P < 0.1\%$) difference between the means of apparent percentage plasmid loss per generation of the two plasmids. Plasmid pKO4.003 shows the highest instability; there is a highly significant ($P < 0.1\%$) difference between the mean percentage plasmid loss per generation of pKO04 and pKO4.003.

The plasmids were checked for structural validity at the end of each experiment (see section 2.13). All digests showed identical banding patterns, suggesting that no large structural changes had occurred over the course of the experiments.

4.4 Discussion

4.4.1 Comparison of the results obtained in this study with those of Williams *et al*. (1998) for plasmids pALA1029, pOG4, pOG04 and pOG4.003.

Both this study and that of Williams *et al*. (1998) have shown pALA1029 to be very stable under batch culture conditions (Fig. 4.2), although there is a highly significant difference ($P < 0.1\%$) between the results from the two studies. This was unexpected

as the experimental results are, in theory, obtained from the same underlying population. This significant difference could be due to some change in the plasmid during the intervening period between studies, or simply because the very low numbers of plasmid-free cells produced during the experiments were not detected in the Williams *et al*. (1998) study. It has been noted that the type of batch culture experiment carried out in this study can be unreliable for stable plasmids (Boe, 1996), and therefore the difference in results can probably be ascribed to experimental error. However, if there has been some change in the stability of pALA1029, this does not cause problems for the current work as the results from this study show that it is still significantly more stable than pOG4, which is all that is required to fulfil the aims.

Comparison of the results obtained in this study and those of Williams *et al*. (1998) (Fig. 4.2) for pOG4 show no significant difference (P > 20%), for pOG04 there is no significant difference (20% > P > 10%), and for pOG4.003 there is also no significant difference (20% > P > 10%). The absence of any significant difference between results indicates that they are part of the same underlying population and therefore suggests that these plasmids have not altered between studies.

4.4.2 Explanations for the different plasmid stabilities observed.

Plasmids pALA1029 and pKO1029 both contain the P7 *par* region, which is responsible for maintaining the prophage of bacteriophage P7 in its host, *E. coli*, as a stable low-copy-number plasmid (Ludtke *et al*., 1989). As a result it would be expected that both plasmids are very stable, and Fig. 4.2 shows that indeed both plasmids have a very low percentage plasmid loss per generation.

Plasmids pOG4 and pKO4 both contain the central control region (*ctl*) from the IncPα plasmid RK2, a plasmid that is capable of stably maintaining itself in almost all Gram-negative bacterial species (Pansegrau *et al*., 1994). The *ctl* region has been shown to decrease the rate of loss of an unstable low-copy-number plasmid (Williams *et al*., 1998). It would therefore be expected that both pOG4 and pKO4 would show high levels of stability, similar to that seen of pALA1029 and pKO1029. The results suggest, however, that plasmids pOG4 and pKO4 have significantly lower stabilities than plasmids pALA1029 and pKO1029.

One reason for this could be the difference in copy number between the plasmids. Where pALA1029 and pKO1029 appear to have a relative copy number of 5, plasmids pOG4 and pKO4 appear to have a relative copy number of 2 (section 3.6). The higher copy number would improve segregational stability as it reduces the chance of plasmid-free cells arising (Nordström & Austin, 1989).

Another reason could simply be that the *ctl* region is not as effective at promoting stability as the P7*par* region. It was discussed in section 1.10.2 that the *ctl* region found in pOG4 and pKO4 (and also pOG4.003 and pKO4.003) is not the only region in plasmid RK2 involved with maintaining stability. Plasmid pOG4 has been further engineered to contain two additional regions, *kil*E and *kil*C, which are normally present upstream of the *ctl* region in plasmid RK2. The insertion of these regions resulted in an increase in stability (Haines, unpublished). However, the proteins encoded by these regions are thought to increase plasmid stability by killing plasmid-free cells as part of a toxin-antidote system (Wilson *et al*., 1997). It would not be advisable to use these altered plasmids in this study for two reasons. Firstly, the partition systems P7*par* and *ctl* work in very similar ways, with two proteins and a *cis*-acting site being involved, and adding a toxin-antidote system would introduce differences between the systems. Secondly, the slightly lower stabilities of plasmids pOG4 and pKO4 are of greater value to this study, as they provide another level of stability.

Neither pOG04 nor pKO04 contain a plasmid partitioning system and therefore at division, each individual plasmid copy has an equal chance of ending up in either of the two daughter cells. If all the plasmid copies end up in one daughter cell then the other daughter cell will be plasmid-free; a likely occurrence with low-copy-number plasmids. The probability (L_{th}) of plasmid-free cells arising in this manner is determined by the copy number of the plasmid, and can be calculated using the formula $L_{th} = (½)^{2n}$, where n is the plasmid copy number (Nordström & Austin, 1989). For example, if the plasmid copy number per cell is two, then the probability of a plasmid free cell arising after division is 6.25%.

The relative plasmid copy number for plasmids pOG04 and pKO04 is approximately two (section 3.6; Williams *et al*., 1998). The results obtained in this study show that pOG04 has a 4.72 ± 2.09% loss rate, and pKO04 2.23 ± 0.66% loss rate, lower than

expected for random partition of a plasmid with a copy number of two per cell. However, it must be remembered that values for plasmid copy number per chromosome will not be the same as values for plasmid copy number per cell due to the presence of multiple chromosomes in actively dividing cells. Therefore, it is likely that the copy number per cell for plasmids pOG04 and pKO04 is higher than two, leading to the higher-than-expected stability of these plasmids.

Plasmids pOG4.003 and pKO4.003 have essentially the same structure as plasmids pOG4 and pKO4 respectively, but in both pOG4.003 and pKO4.003 the O_B3 site within the *ctl* partition region has been mutated. Mutation of the O_B3 site seems to prevent separation of the plasmids, which results in transfer into daughter cells of joined plasmid copies, effectively reducing the copy number and leading to high plasmid instability (section 1.10.2; Williams *et al*., 1998). The end result is a plasmid partition system which is less effective than random segregation, which is the situation seen in plasmids pOG4.003 and pKO4.003.

4.4.3 Mathematical analysis of the reasons for the varied plasmid stability levels observed

Applying mathematical models to experimental data assists in analysis, and can often identify the causes of unexpected results. There have been criticisms of the way in which models are applied to all plasmid stability experiments, as these models assume that all plasmid-bearing cells have the same copy number, growth rate and probability of production of plasmid-free cells (Kim & Shuler, 1990). There is also an assumption made that the production of plasmid-free cells remains constant throughout the growth phases of a culture, that is, the occurrence of plasmid segregation will not vary over the course of the experiment. While this may not necessarily be the case; changes in plasmid copy number or mutations in either the plasmid or host cell would affect loss rates; it must be assumed to be constant for the success of the application of mathematical modelling (Davidson *et al*., 1990). In the following section, the mathematical model published by Cooper *et al*. (1987) will be applied to the results obtained in this study to assist in analysis.

The important factors involved are the segregational instability of the plasmid, *R*, and the difference in growth rate between plasmid-free and plasmid-bearing cells, $d\mu$ (Cooper *et al*., 1987). The value of $d\mu$ is an indication of the effect of plasmid burden on the host cell. For all the plasmids studied, the plasmid burden is low because they are relatively small plasmids, they have a low copy number, and they are not producing large proteins. Due to the similarities of the plasmids, $d\mu$ is unlikely to vary greatly between them. On the other hand, *R* will change dramatically between plasmids because of the different partition systems as described in section 4.4.2. For all the plasmids studied, the interrelationship between these two factors will be determined and discussed to assist in the interpretation of the results. The actual values of $d\mu$ and *R* have not been determined through these experiments and therefore the relationships between $d\mu$ and *R* described below are presumptive.

For plasmids pALA1029 and pKO1029, *R* is likely to be low because of the presence of an effective partition system. In contrast to this low value of *R*, the value of $d\mu$ may be comparatively large, therefore described as $d\mu \gg R$. In this situation the number of plasmid-free cells at the start of the population is low because of the low value of *R*, but when plasmid-free cells are produced they will multiply more effectively than the plasmid-bearing cells and will increase exponentially within the population. This situation is shown graphically in Fig. 4.3.

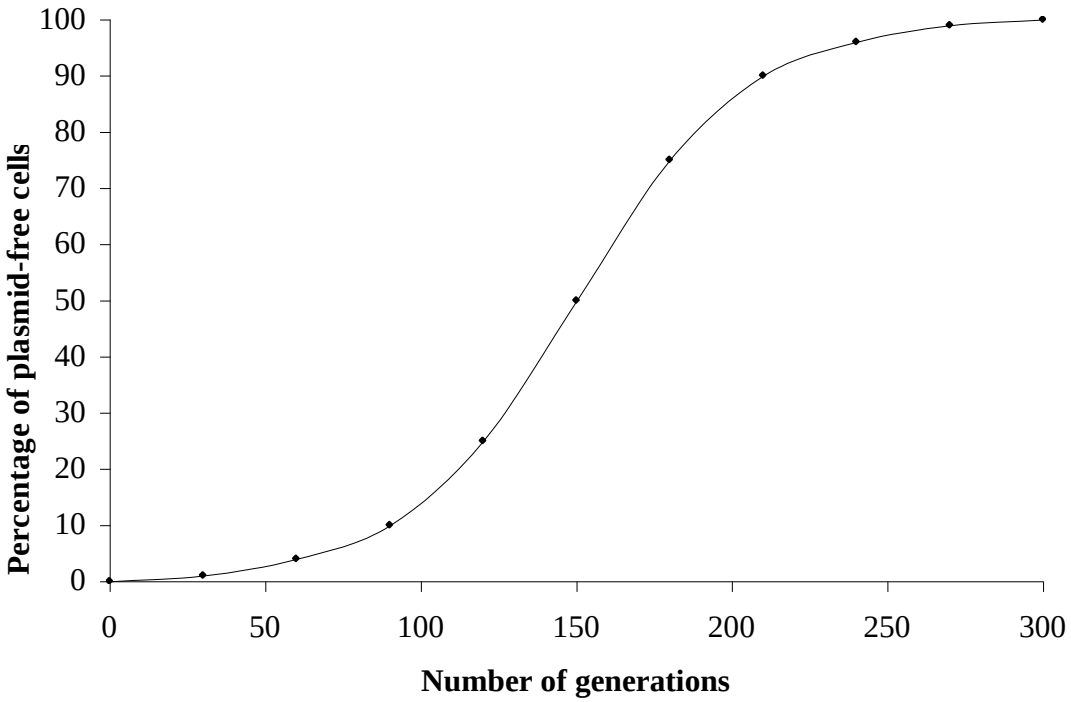


Fig. 4.3: The mathematical model illustrating the increase in plasmid-free cells over generations of growth in a situation described as $d\mu \gg R$ (Cooper *et al*., 1987)

The exponential increase of plasmid-free cells begins slowly as few plasmid-free cells are produced when the plasmid contains an effective stability system. As a result, plasmid-free cells are not a significant proportion of the population until after approximately 50 generations (Cooper *et al*., 1987). As the experiments carried out in this study and by Williams *et al*. (1998) followed only 25 generations, any exponential increase would not have been observed. It is a limitation of small volume batch culture experiments that growth can only be observed over approximately 25 generations, as after this point nutrient limitation and toxic waste build-up leads to the death phase of the culture.

The decrease in stability for the plasmids pOG4 and pKO4 as compared to pALA1029 or pKO1029 is almost certainly caused by a change in *R*. It is known that the partition system used within these plasmids is not fully functional (section 4.4.2), and therefore the segregational loss rate will be higher. It is likely that $d\mu$ will have altered because

of a decreased copy number, but this change will probably be insignificant when compared to the assumed increase of *R*. As a result the situation may be best described as $|d\mu| \leq R$. In this situation indicates there is a steady and relatively linear increase in the number of plasmid-free cells throughout the experimental time. This is shown in a graphical form in Fig. 4.4.

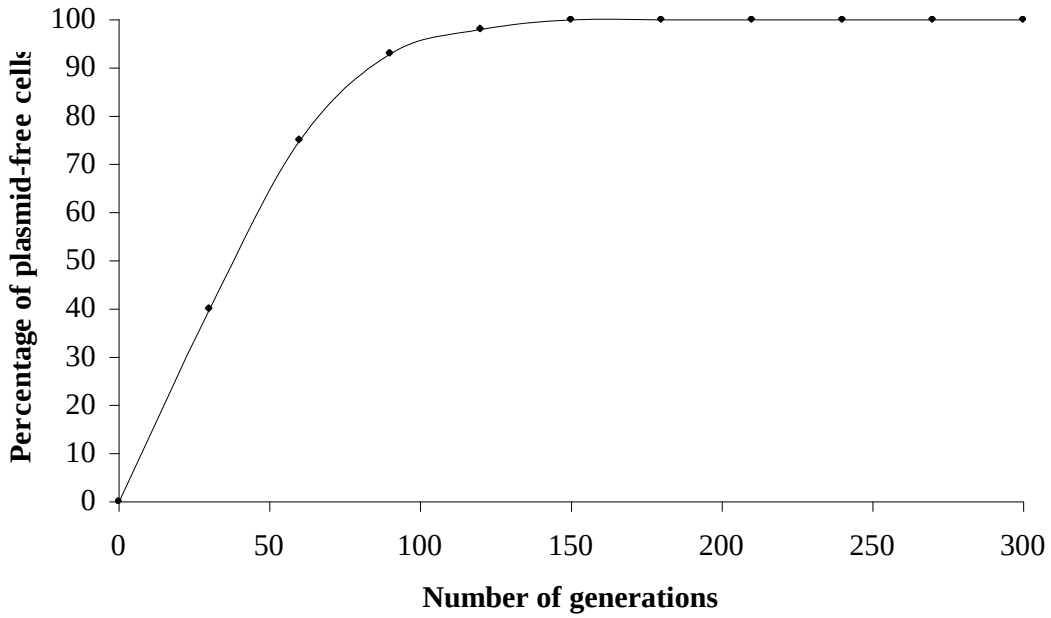


Fig. 4.4: The mathematical model illustrating the increase in plasmid-free cells over generations of growth in a situation described as $|d\mu| \leq R$ (Cooper *et al.*, 1987)

Plasmid-free cells are being produced relatively regularly throughout the experiment, and while these do not multiply any faster than the plasmid-bearing cells, they will still increase through division. The important point in this situation is that the segregational instability is a more important cause of an increase in plasmid-free cells than the ability of the plasmid-free cells to grow.

The further decrease in the stability of plasmids pOG04 and pKO04 is again likely to be due to another increase in the value of *R*. Random segregation of plasmid copies at a low copy number is not an effective method of ensuring plasmid stability, and as the value of *R* increases, so the value of $d\mu$ becomes even less significant. In these

plasmids $d\mu$ is also likely to be smaller than that of pOG4 and pKO4, because the plasmids are smaller and produce fewer proteins because of the lack of a partition system. Therefore the model situation is likely to be $|d\mu| \leq R$.

Another increase in the value of R will also be the cause of increased stability in the plasmids pOG4.003 and pKO4.003. In this case, the value for $d\mu$ is likely to be identical to that of plasmids pOG4 and pKO4, as there is no difference in plasmid size. There may be a subtle alteration in the levels of gene expression caused by the deletion of the O_B3 site, as there would be an increase in the amount of available KorB protein (Lukaszewicz *et al*., 2002). However, this would probably not cause a large change in the value of $d\mu$, and any change would be insignificant when compared to the large value of R. Therefore the model situation here is also likely to be $|d\mu| \leq R$.

4.4.4 Comparison of the stabilities of the ampicillin-resistance conferring plasmids with those of the chloramphenicol-resistance conferring plasmids under batch culture conditions

The above discussions have concentrated on the stability of each individual plasmid, and have shown that the order of increasing instability of the ampicillin-resistance conferring plasmids is the same as that seen for the chloramphenicol-resistance conferring plasmids. In other words, both pKO1029 and pALA1029 are the most stable plasmids and pOG4.003 and pKO4.003 are the least stable plasmids. However, Fig. 4.2 indicates that the chloramphenicol-resistance conferring plasmids are, in general, more stable than their parental ampicillin-resistance conferring plasmids.

According to the results obtained in this study there is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid loss per generation for pKO1029 and pALA1029. There is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid loss per generation for plasmids pKO4 and pOG4, and no significant difference ($P > 20\%$) between plasmids pKO04 and pOG04. There is, however, a highly significant difference ($P < 0.1\%$) between the mean apparent percentage plasmid loss per generation for plasmids pKO4.003 and pOG4.003.

The lack of significant differences between the results is probably due to a lack of replication of the batch culture experiments on the ampicillin resistance conferring plasmids in this study. In order to compare the results obtained for the chloramphenicol resistance-conferring plasmids with those for the ampicillin resistance-conferring plasmids, the statistically significant results obtained by Williams *et al*. (1998) will be used. Statistical tests have shown that the plasmids have not altered between the Williams *et al*. (1998) study and this study, with the exception of plasmid pALA1029, which is likely to be due to the inaccuracies of batch culture experiments for studying stable plasmids rather than changes in the stability system (section 4.4.1).

When comparing the results obtained by Williams *et al*. (1998) for the ampicillin resistance-conferring plasmids and the results for the chloramphenicol resistance-conferring plasmids obtained in this study, the following differences can be observed: the mean apparent percentage plasmid loss per generation for pKO1029 is significantly higher ($1\% > P > 0.5\%$) than the result for pALA1029. The percentage plasmid loss per generation for pKO4 is significantly ($1\% > P > 0.5\%$) lower than that for pOG4, the percentage plasmid loss per generation for pKO04 is significantly ($P < 0.1\%$) lower than that for pOG04, and the percentage plasmid loss per generation for pKO4.003 is significantly ($P < 0.1\%$) lower than that for pOG4.003. This difference between the results obtained for pKO4.003 and pOG4.003 was also seen to be statistically significant ($P < 0.1\%$) when the results obtained in this study were compared.

This leads to the conclusion that the addition of the fragment of DNA containing the chloramphenicol-resistance gene has increased the stability of the plasmid when compared to its parent plasmid that confers ampicillin resistance, with the exception of plasmid pKO1029. Again this exception is likely to be due to the inaccuracies of batch culture experiments for plasmid stability studies when the stability is high. A comparison of results obtained during this study suggests that there is no significant difference ($P > 20\%$) between the mean apparent percentage plasmid stabilities of plasmids pKO1029 and pALA1029.

It was not expected that addition of the fragment of DNA from plasmid pJL3-1974 would alter the stability levels of the plasmids, although the possibility of increased instability due to the extra plasmid burden had been considered.

This increased stability could be due to unseen alteration of the chloramphenicol-resistance-conferring plasmids during their production, although this is unlikely as mutations must have occurred simultaneously producing a similar effect in each of the four plasmids.

Therefore, the extra DNA that contains the chloramphenicol-resistance gene, the only known difference between the two sets of plasmids, is likely to be the cause of the increase in stability due to an decrease in the *R* or $d\mu$ values of the plasmids. A decrease in the value of *R* would reduce the number of plasmid-free cells appearing, leading to higher plasmid stability. A decrease in the $d\mu$ value would mean that the plasmid-free cells could not multiply quickly enough to overwhelm the remaining plasmid-bearing cells, also leading to a higher plasmid stability. Several theories as to why the *R* or $d\mu$ values might have decreased are described in the following sections.

4.4.4.1 A decrease in *R* caused by the addition of an extra promoter

The section of DNA ligated into the plasmids contains a promoter for the chloramphenicol resistance gene, and it is possible that the terminator is not functioning efficiently. As a result, there could be increased expression of the downstream plasmid partition genes, leading to a more effective partitioning phenotype and therefore a decrease in the value of *R*. However, this is unlikely to be the case, as plasmid pKO04 shows increased stability as compared to pOG04, yet it does not contain a partition system. Also, it has been shown that simultaneous overexpression of the *korB* and *incC* genes within the *ctl* system is toxic to *E. coli* cells (Rosche *et al*., 2000), and overexpression of *incC* will increase the plasmid loss rate (Jagura-Burdzy *et al*., 1999). However, the possibility of increased promoter activity on other genes within the plasmids cannot be ruled out, and this may cause a difference in stability (Katayama & Nagata, 1990).

4.4.4.2 A decrease in *R* caused by an increase in plasmid copy number

The effects of plasmid copy number on plasmid stability have been widely discussed (Nordström & Austin, 1989), and it has been noted that increasing the copy number of small plasmids improves stability (Allen & Blaschek, 1990). The Southern blots carried

out (section 3.6) were not quantified, and as a result cannot be used to definitively state whether plasmids pKO1029, pKO4, pKO04 and pKO4.003 have an increased plasmid copy number. Plasmid pKO04 may have an increased plasmid copy number as the plasmid bands did appear slightly darker relative to the chromosomal band (Fig. 3.9). However, there has not been a dramatic increase in copy number for any of the plasmids; consequently a decrease in the value of *R* caused by an increased copy number is likely to be insignificant.

4.4.4.3 An apparent decrease in *R* caused by transfer of the chloramphenicol resistance gene to the chromosome

The insert used to add chloramphenicol resistance to the plasmids is composed of a large portion of transposon Tn*9*. This composite transposon is flanked by two IS1 insertion sequences (Alton & Vapnek, 1979), and IS1 sequences can also be found in the chromosome of *E. coli* (Clugston & Jessop, 1991). While the IS1 elements within the insert are truncated and therefore no longer functional, homologous recombination between the IS1 sequences on the plasmid and chromosome could have occurred during the course of the experiments (Rohmer *et al*., 2003). Recombination would have led to transferral of chloramphenicol resistance to the chromosome, leading to inherent resistance to chloramphenicol even in the absence of a plasmid, and an apparent decrease in the value of *R*. However, all chloramphenicol resistant cells tested at the end of the experiments (section 2.13) were found to contain a plasmid. While this does not rule out the possibility of chromosomal integration, if integration is taking place it is occurring infrequently, and would therefore have had limited effect on the apparent stability of the plasmid.

4.4.4.4 An apparent decrease in *R* due to the different modes of action of the antibiotics

The original reason for constructing the chloramphenicol-resistance-conferring plasmids was to determine whether the different modes of action of ampicillin and chloramphenicol would have an effect on plasmid stability. As plasmid stability does appear to have been affected, it is worth considering the possibility that the different antibiotics are responsible for this. It must be remembered that, for batch culture, the

main part of the experiment is carried out in the absence of antibiotics. However, media are supplemented with antibiotics for replica plating purposes, and it is during this stage that the different antibiotics could affect the results obtained.

For these experiments, an assumption is made that an antibiotic resistant colony is wholly made up of plasmid-bearing cells. However, while the single cell that formed an antibiotic resistant colony must have contained a plasmid, if that plasmid is unstable then there is a chance that some of the daughter cells produced within the colony would be plasmid-free, and in the absence of antibiotics would be able to grow.

When this type of mixed colony is replica plated onto ampicillin-containing media, any plasmid-free cells within the colony would die, leaving only plasmid-bearing cells to grow. If the colony had contained a large number of plasmid-free cells, then the number of plasmid-bearing cells remaining may not be large enough to form a noticeable colony on the plate after overnight growth. As a result, the colony would have been recorded as sensitive to antibiotics, consequently leading to a higher percentage plasmid loss per generation.

On the other hand, when a mixed colony is plated onto chloramphenicol, any plasmid-free cells will stop growing, but will not necessarily be killed. Meanwhile, the plasmid-bearing cells will produce CAT, which will reduce the concentration of the chloramphenicol in the media. It is possible that the concentration of chloramphenicol around the colony may decrease enough to allow the plasmid-free cells to begin growing again, subsequently producing a clearly visible colony after overnight growth. Therefore a lower percentage plasmid loss per generation will be recorded.

The production of mixed colonies would become more frequent as the plasmid stability decreased. For stable plasmids pALA1029 and pKO1029, very few plasmid-free cells are formed and therefore few mixed colonies would arise. This may explain why the stability levels of these two plasmids are similar. On the other hand, both plasmids pKO4.003 and pOG4.003 are unstable, leading to the frequent production of mixed colonies. If the antibiotics affected these mixed colonies as described above, this may explain why pKO4.003 is seen to be more stable than pOG4.003.

Structural validity checks on the plasmids at the end of the experiment (section 2.13) would not identify mixed colonies, as the plasmid DNA is amplified during the process. Therefore, if only a single cell within the colony contained a plasmid, the agarose gel would still show that a plasmid was present.

4.4.4.5 A decrease in $d\mu$ caused by a difference in levels of expression of ß-lactamase and CAT

The suggestion that the increased plasmid stability seen in the chloramphenicol-resistance-conferring plasmids is due to a reduction in plasmid burden seems very unlikely; as the immediate assumption is that the plasmid burden would be higher due to their increased size. However, DNA replication does not require much host cell energy (Cooper *et al*., 1987), and therefore the additional DNA present in the chloramphenicol-resistance-conferring plasmids should not noticeably affect plasmid stability. On the other hand, protein production does require a large amount of energy and materials from the host cell (Cooper *et al*., 1987; Lenski & Nguyen, 1988), and therefore if there is a difference in the quantities of ß-lactamase and CAT produced, then this may affect $d\mu$.

For $d\mu$ to be reduced in the cultures containing chloramphenicol-resistance-conferring plasmids, the amount of CAT produced must be lower than the amount of ß-lactamase produced by the ampicillin-resistance-conferring plasmids. The promoters control regulation of the amount of protein produced, but the natures of the promoters controlling the ß-lactamase and CAT genes have been very difficult to ascertain.
It appears that the ß-lactamase gene in plasmids pALA1029, pOG4, pOG04 and pOG4.003 is under the control of two promoters, the P1 and P3 *E. coli* promoters (VectorDB, 1997). The P1 promoter is a strong constitutive promoter (Andreeva *et al*., 2000) suggesting that the large ß-lactamase protein is being constantly produced in these plasmids.

The CAT gene also appears to be under the control of a constitutive promoter, but a much weaker one that is modulated by the binding of CAP/cAMP complexes, similar to that seen in the *lac* operon (Le Grice & Matzura, 1981; Le Grice *et al*., 1982). Therefore it is possible that the amount of CAT produced is lower than the amount of

ß–lactamase produced, which would reduce the comparative plasmid burden on the host cells. As a result, the growth rate of the plasmid-bearing cells would be closer to that of the plasmid-free cells, which would prevent plasmid-free cells outgrowing the plasmid-bearing cells, leading to an apparent increase in plasmid stability.

4.4.5 Experiments that could provide further insights into the causes of the differences in plasmid stability

None of the reasons for the increased stabilities of the plasmids pKO1029, pKO4, pKO04 and pKO4.003 discussed in section 4.4.4 can be categorically proved with the amount of data currently available. Other experiments that could be carried out to gain further insights are described below.

New plasmids could be constructed that contained the chloramphenicol-resistance gene inserted in a way that prevented disruption of the ampicillin-resistance gene. Batch culture experiments could be carried out on these plasmids in the presence of ampicillin and the results compared to the results from batch culture experiments carried out on pALA1029, pOG4, pOG04 and pOG4.003. If the stabilities were very similar, this would suggest that the plasmid burden has not been increased by the addition of CAT gene, adding weight to the hypothesis described in section 4.4.4.5. If the stabilities of the new plasmids were higher than those of pALA1029, pOG4, pOG04 and pOG4.003, then the hypotheses described in sections 4.4.4.1 through 4.4.4.4 are more likely to be true.

A more simple further experiment would involve carrying out batch culture experiments on plasmids pKO1029, pKO4, pKO04 and pKO4.003 where the insert is in the opposing (clockwise) orientation. If the stabilities of these plasmids reverted to those seen for plasmids pALA1029, pOG4, pOG04 and pOG4.003, this may suggest that the CAT promoter may be affecting genes throughout the plasmid (section 4.4.4.1).

The use of quantitative RT-PCR (reverse transcriptase polymerase chain reaction) could be used to highlight a differential expression of the ß-lactamase and CAT proteins (section 4.4.4.5). This technique would determine the amount of mRNA produced from

these two genes, showing whether CAT is indeed produced at a low level in the absence of chloramphenicol.

4.5 Conclusion

The results obtained from batch culture experiments described in this chapter show that different plasmid stability systems can affect the production of plasmid-free cells. This is clearly seen when comparing a stable plasmid, such as pALA1029, with a plasmid containing a defective stability system, such as pOG4.003. The batch culture technique is fast and simple, which can highlight these differences efficiently. Even though the experiments on the ampicillin resistance-conferring plasmids were only repeated a minimal number of times in this study; conclusions could be drawn when compared to the results obtained by Williams *et al*. (1998), which were acquired from a significantly greater number of experimental repeats.

The production of a range of plasmids conferring chloramphenicol resistance further enhanced the results obtained. Again, the differences in plasmid stability systems were seen to affect the number of plasmid-free cells produced over several generations of growth, indicating that these experiments can quickly highlight differences in plasmid stability, regardless of the nature of the plasmid. This work also showed that the addition of the CAT gene increased plasmid stability when compared to plasmids conferring ampicillin resistance, although the cause of this effect cannot be identified at the present time.

CHAPTER FIVE – MEASUREMENT OF THE STABILITY OF PLASMIDS pALA1029, pOG4, pOG04, pOG4.003, pKO1029, pKO4, pKO04 AND pKO4.003 IN STEADY STATE CHEMOSTAT CULTURE

5.1 Introduction

The measurement of plasmid stability through batch culture experiments, as carried out in chapter four, is the most commonly used and published method (Kim & Blaschek, 1989; Roberts & Helinski, 1992; Easter *et al.*, 1998). This is because it is a simple method that can provide data relatively quickly. However, the main aim of this study was to quantify the benefit of destabilising an antibiotic-resistance plasmid present within bacterial cells causing a clinical infection, a situation that cannot be easily commented on from the results of batch culture experiments.

Bacterial infections begin with a small number of bacterial cells entering a susceptible site, followed by colonisation and multiplication of the organisms. At this point, in most patients, the immune system comes into play, leading to the classic symptoms of infection such as inflammation. In many cases the immune system alone will be able to eradicate the infection, assuming that the host is in relatively good health otherwise. Patients infected with life-threatening pathogens, or patients that do not have an effective immune system, may require additional assistance from medication in order to clear the infection, antibiotics being the most commonly prescribed. An infection that develops rapidly and only lasts for a short period of time is termed an acute infection.

A chronic infection usually develops from an acute infection that could not be completely cleared by the immune system or medication (Reade *et al*., 1998). It can also be described as an infection that develops slowly and lasts for a long period of time. The bacteria remain within the body at low levels for months or years and in some cases the infection may never be completely eradicated. This usually occurs because the immune system is not functioning correctly, for example in AIDS patients, or where additional treatment with antibiotics has failed because the infecting organism has resistance to antibiotics.

If antibiotic resistance is the cause of the persistence of a chronic infection, then reducing the resistance may improve the chances of treatment, resulting in the clearing of the infection. One possible way of reducing the antibiotic resistance within the cell population is by destabilising the transfer of antibiotic-resistance plasmids from parent to daughter cells during replication. This destabilisation of plasmid inheritance would produce plasmid-free cells that would be susceptible to antibiotic treatment (section 1.8). However, the amount of destabilisation required to produce enough plasmid-free cells to provide an observable benefit has not been previously determined.

An experimental system that would accurately model the conditions found in a patient with a clinical infection would therefore be of benefit for this study. It would allow determination of the effects of destabilisation on the antibiotic sensitivity of a culture growing under conditions similar to those seen in an infection.

Previously, most experiments requiring a model of a human system have used animal models in order to obtain the data required. While this type of experiment is important for detailed studies, a preliminary investigation such as that carried out in this study would benefit from a less complicated model. Macfarlane *et al*. (1998) used a continuous chemostat culture system in order to model the human colon. This validated model was used in a more recent study (Freeman *et al*., 2003), the aim of which was to determine how the treatment of *Clostridium difficile* with antibiotics is affected by the normal microflora present in the human gut. One conclusion of the study was that the chemostat model provided conditions homologous to those found in an *in vivo* situation.

The chemostat was first described in 1950 as "a device for keeping a bacterial population growing at a reduced rate over an indefinite period of time" (Novick & Szilard, 1950). A chemostat is a vessel containing a culture of organisms into which fresh medium added at a constant rate. The vessel is stirred and air is pumped into it in order to keep the culture suspended. The culture volume is kept constant by an overflow pipe, which removes exhausted medium, living cells and cellular debris (Dykhuizen & Hartl, 1983), and as a result, the bacterial population will be maintained at a specific population density (Herbert *et al*., 1956). The organisms are kept at a reduced growth rate by a growth-limiting factor, the concentration of which will determine the cell density in the chemostat (Novick & Szilard, 1950). All the factors

controlling growth in the chemostat can be defined mathematically, and the basic mathematical formulae are described below (Pirt, 1975).

The dilution rate of the chemostat is determined by the inflow of fresh medium into the culture vessel.

(1) Dilution rate = rate of medium inflow ⁄ volume of the vessel

Or $D = F / V$

The growth of the culture in the chemostat is defined as:

(2) specific growth rate of biomass = change in biomass ⁄ change in time

Or $\mu x = dx / dt$

However, in chemostat culture, cells are also lost through overflow into the waste tank. The dilution rate of the biomass is defined as:

(3) dilution of biomass = change in biomass ⁄ change in time

Or $Dx = - (dx / dt)$

As cell growth is limited by the amount of substrate present in the chemostat vessel, the cells cannot divide unless fresh media enters the vessel. However, at the same time, as fresh media enters an equivalent volume of culture is expelled through the overflow.

In steady state chemostat culture the two equations (2) and (3) can be combined.

(4) growth of biomass = dilution of biomass

Or $\mu x = Dx$

Or $\mu = D$

The dilution rate therefore controls the growth rate of the culture due to substrate limitation. If the dilution rate is increased, the growth rate will increase ensuring that the biomass remains at a constant. This holds true until the dilution rate increases above the maximum specific growth rate (μ_{max}) of the culture. This point is known as the critical dilution rate (D_{crit}), and the biomass in the vessel will reduce because the cells cannot replicate quickly enough to replace those lost through the overflow. Substrate limitation is no longer a factor, and washout occurs.

However the μ_{max} can change depending on the temperature, concentration of the growth-limiting factor, amount of oxygen available and many other variables. Therefore μ_{max} can only be defined when the conditions of the chemostat culture are also specifically defined.

Calculation of the generation time of the culture at a specified dilution rate in the absence of washout can be determined by using an exponential doubling constant ($e^{\mu t}$).

(5) Biomass at a given time = biomass at start of experiment $\times e^{\mu t}$

Or $x_t = x_o \times e^{\mu t}$

For one generation of bacterial growth when the time of the experiment (t) is the same as the doubling time (T) the biomass will double.

(6) biomass at time $_t$ / biomass at time $_0 = 2$

Or $x_t / x_o = 2$

Therefore $e^{\mu t} = 2$ when the chemostat is in steady state. Equation (5) can be simplified to determine doubling time in a specific steady state condition.

(7) Doubling time = natural log of 2 / growth rate

Or $T = \log_e 2 / \mu$

When the biomass within the culture vessel remains constant, the situation is known as 'steady state', and has similarities to a chronic infection. In both cases the bacterial cell population is continually multiplying; however, population growth in a chemostat is being limited by washout into the waste vessel, whereas in a chronic infection the 'washout' is caused by the partial eradication of cells due to the effects of the immune system. This model of chronic infection can include exposure of the culture to antibiotics in order to simulate the effects of antibiotic treatment in an infected patient.

There are many reasons why the chemostat is a good experimental model for a bacterial infection, but there are also limitations to its use. Laboratory cultures can never accurately imitate conditions found naturally within the environment (Kovárová-Kovar & Egli, 1998). Therefore, while chemostat culture provides more similarities than batch culture, it cannot be assumed that this as accurate a model of a clinical infection as

animal models would be.

The measurement of plasmid stability under steady state chemostat culture conditions is not often undertaken, although many mathematical models are based on plasmid stability in chemostat culture, which can assist in interpretation of the data obtained (Cooper *et al*., 1987). The question to be answered by this section of work is, therefore, whether any of the plasmids being studied have a large enough plasmid loss rate to cause a washout of the chemostat culture, thereby indicating whether or not plasmid destabilisation could be effective in clearing chronic clinical infections.

5.2 Method

The chemostat culture experiments were carried out as described in section 2.13.3. A chemostat culture is considered to have reached steady state after 2.5 volumes of fresh medium has passed through the vessel. This means that the equilibrium between plasmid-free and plasmid-bearing cells in these experiments should have been established after approximately 12½ hours, as fresh media was entering the vessel containing one-litre of medium at a rate of 0.2 litres per hour.

At least five individual chemostat cultures were run for each plasmid, using a fresh transformant each time. Samples were taken from the chemostat each day for five days and plated onto LB agar (section 2.3.1). The sensitivity or resistance of each colony to antibiotics was determined using replica plating (section 2.13.3). These results were plotted on a graph showing the change in antibiotic sensitivity over the number of generations of the culture (Figs. 5.1 to 5.13). The results from each plasmid for percentage sensitivity obtained after steady-state had been reached (from the second sample) were used to calculate a mean percentage sensitivity to antibiotics at equilibrium for each plasmid. 95% confidence limits were calculated (section 2.14.1) on this mean, and these results were used to produce the bar chart found in Fig. 5.14. In order to determine the statistical significance between the results obtained, the Student *t*-test was used (section 2.14.2).

5.3 Results

5.3.1 The percentage sensitivity to ampicillin of individual steady-state chemostat cultures of *E. coli* C2110 containing pALA1029, pOG4, pOG04 or pOG4.003.

The results obtained from chemostat experiments of *E. coli* C2110 containing the four ampicillin resistance-conferring plasmids are shown in Tables 5.1 to 5.4 and Figs. 5.1 to 5.6. For the purpose of comparison, four of the graphs produced use the same scale for percentage sensitivity, showing 0 to 100% sensitivity to ampicillin (Figs. 5.2, 5.4, 5.5 and 5.6). The other two graphs (Figs. 5.1 and 5.3) show the results for plasmids pALA1029 and pOG4 using a smaller scale for percentage sensitivity, allowing the individual results to be observed more clearly.

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	69	0	0
	18	5.19	45	0	0
	42	12.1	62	1	1.61
	65.8	19.0	92	1	1.09
	90.1	26.0	111	1	0.91
2	0	0	97	2	2.06
	18	5.19	48	1	2.08
	42	12.1	152	3	1.97
	65.8	19.0	79	2	2.53
	90.1	26.0	123	4	3.25
3	0	0	186	1	0.54
	19.5	5.63	72	0	0
	44.4	12.8	111	1	0.91
	68.6	19.8	155	1	0.65
	91.5	26.4	165	1	0.61
4	0	0	121	0	0
	19.5	5.63	40	0	0
	44.4	12.8	86	0	0
	68.6	19.8	63	0	0
	91.5	26.4	114	0	0
5	0	0	157	2	1.27
	19.2	5.54	97	2	2.06
	48.2	13.9	54	1	1.85
	69.6	20.1	45	1	2.22
	89.4	25.8	53	1	1.89
6	0	0	165	0	0
	19.2	5.54	35	0	0
	48.2	13.9	95	0	0
	69.6	20.1	73	0	0
	89.4	25.8	43	0	0
7	0	0	112	0	0
	16.5	4.76	99	0	0
	41.9	12.1	87	0	0
	65.5	18.9	96	0	0
	85.2	24.6	56	0	0

Table 5.1: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pALA1029

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	61	0	0
	30.2	8.73	119	2	1.68
	55.4	16.0	48	2	4.17
	77.6	22.4	250	12	4.80
	99.4	28.7	118	5	4.24
2	0	0	50	2	4.00
	17.3	4.98	44	1	2.27
	42.3	12.2	77	1	1.30
	68.3	19.7	123	4	3.25
	90.1	26.0	114	2	1.75
3	0	0	46	2	4.35
	18.1	5.21	44	2	4.55
	44.4	12.8	57	3	5.26
	70.3	20.3	106	6	5.66
	89.4	25.8	135	8	5.93
4	0	0	46	2	4.35
	18.1	5.21	40	2	5.00
	44.4	12.8	123	6	4.88
	70.3	20.3	249	9	3.61
	89.4	25.8	158	7	4.43
5	0	0	73	0	0
	16.5	4.76	40	1	2.50
	41.9	12.1	121	4	3.31
	65.5	18.9	228	8	3.51
	85.2	24.6	113	4	3.54

Table 5.2: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG4

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	55	27	49.1
	22.0	6.35	57	39	68.4
	52.3	15.1	57	45	79.0
	76.6	22.1	44	34	77.3
	99.4	28.7	52	40	76.9
2	0	0	46	20	43.5
	22.0	6.35	40	18	45.0
	52.3	15.1	42	28	66.7
	76.6	22.1	76	55	72.4
	99.4	28.7	30	20	66.7
3	0	0	115	43	37.4
	23.0	6.64	247	128	51.8
	48.5	14.0	224	115	51.3
	74.5	21.5	63	40	63.5
	89.4	25.8	70	43	61.4
4	0	0	31	13	41.9
	19.0	5.48	196	86	43.9
	41.2	11.9	108	63	58.3
	67.6	19.5	104	72	69.2
	89.1	25.7	31	21	67.7
5	0	0	180	102	56.7
	19.0	5.48	118	68	57.6
	41.2	11.9	47	37	78.7
	67.6	19.5	79	59	74.7
	89.1	25.7	47	34	72.3
6	0	0	93	46	49.5
	20.9	6.64	34	18	52.9
	41.6	11.9	33	24	72.7
	66.9	19.3	46	33	71.7
	90.4	25.8	50	36	72.0

Table 5.3: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG04

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	54	18	33.3
	16.9	4.89	72	3	4.17
	29.0	8.36	63	4	6.35
	52.7	15.2	61	3	4.92
	78.7	22.7	34	2	5.88
2	0	0	43	16	37.2
	18.0	5.19	36	12	33.3
	41.9	12.1	45	7	15.6
	65.8	19.0	43	9	20.9
	90.1	26.0	82	10	12.2
3	0	0	68	20	29.4
	23.0	6.64	64	12	18.8
	41.2	11.9	85	11	12.9
	66.9	19.3	48	7	14.6
	89.4	25.8	86	12	14.0
4	0	0	36	30	83.3
	8.00	2.31	35	20	57.1
	25.0	7.22	42	4	9.52
	50.0	14.4	62	4	6.45
	75.9	21.9	56	5	8.93
5	0	0	48	16	33.3
	8.00	2.31	34	4	11.8
	25.0	7.22	56	6	10.7
	50.0	14.4	32	4	12.5
	75.9	21.9	36	4	11.1
6	0	0	60	12	20.0
	22.0	6.35	40	8	20.0
	50.6	14.6	77	11	14.3
	74.5	21.5	59	10	17.0
	89.4	25.8	47	7	14.9
7	0	0	64	48	75.0
	22.0	6.35	102	9	8.82
	50.6	14.6	138	10	7.25
	74.5	21.5	54	4	7.41
	89.4	25.8	32	3	9.38
8	0	0	53	23	43.4
	18.0	5.19	40	9	21.4
	41.9	12.1	64	12	18.8
	65.8	19.0	54	12	22.2
	90.1	26.0	40	8	20.0

Table 5.4: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG4.003

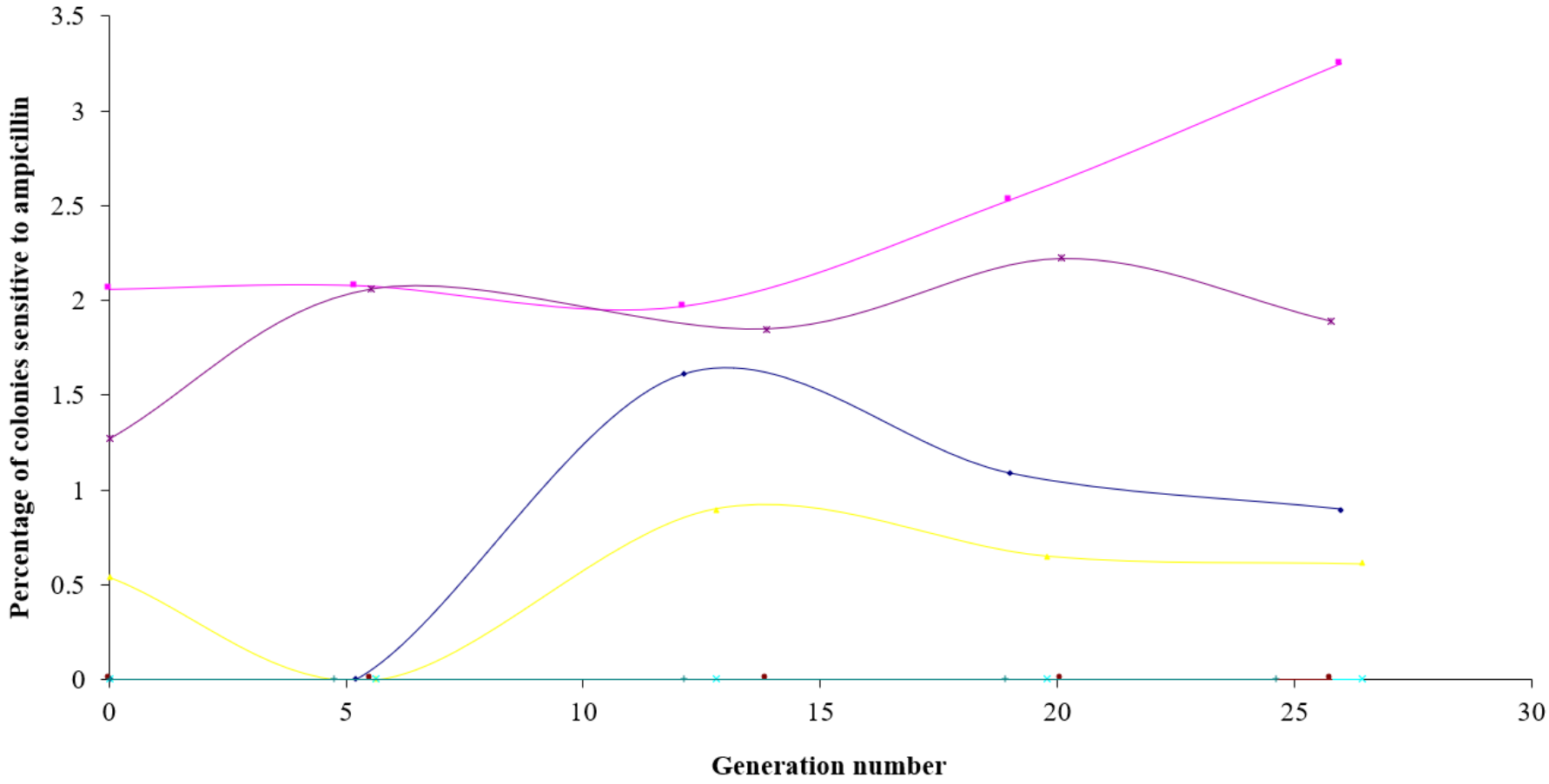


Fig. 5.1: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG1029, plotted using a small scale for percentage sensitivity for clarity**

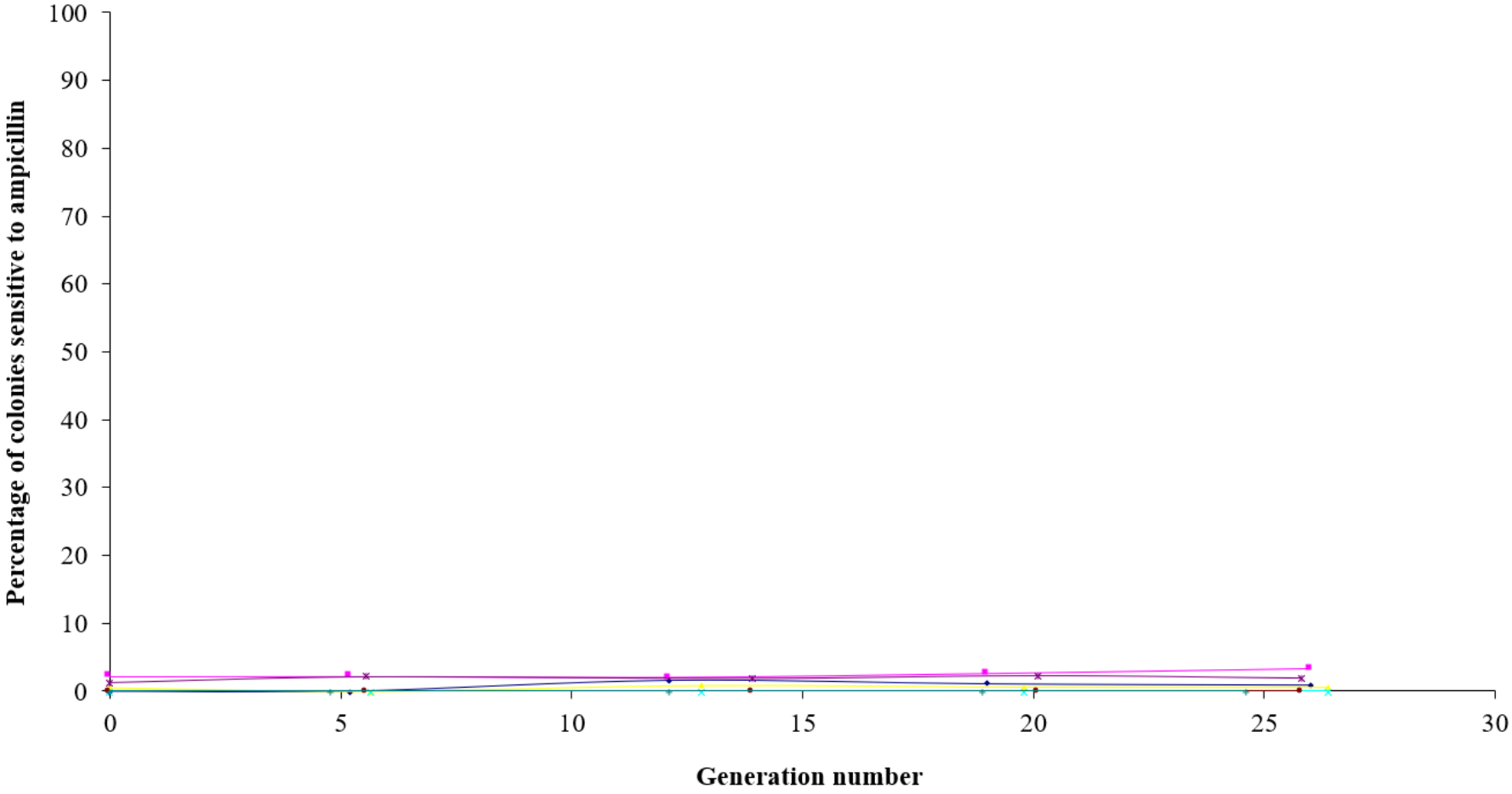


Fig. 5.2: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG1029, plotted using a large scale for percentage sensitivity for comparison**

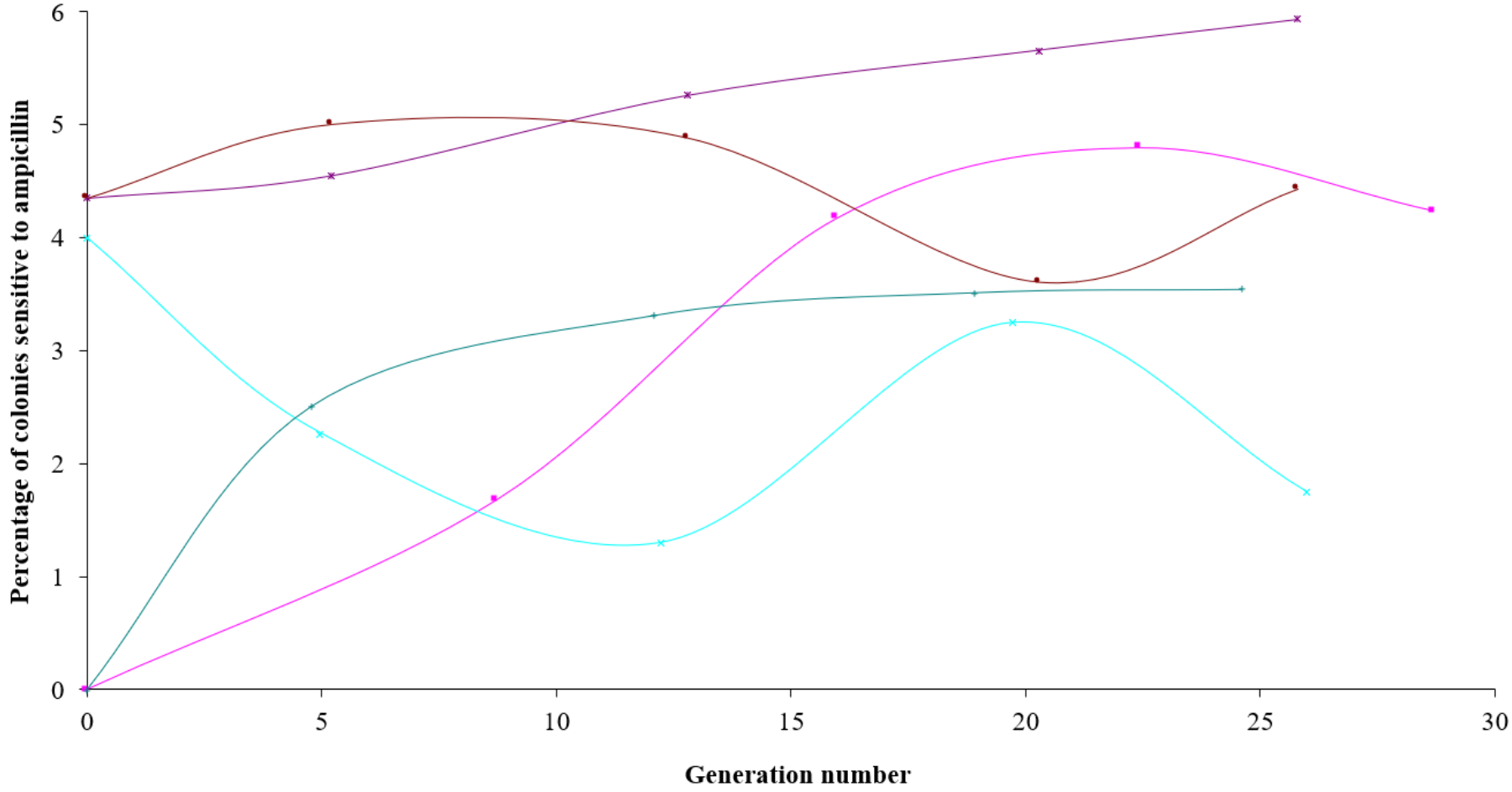


Fig. 5.3: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG4, plotted using a small scale for percentage sensitivity for clarity**

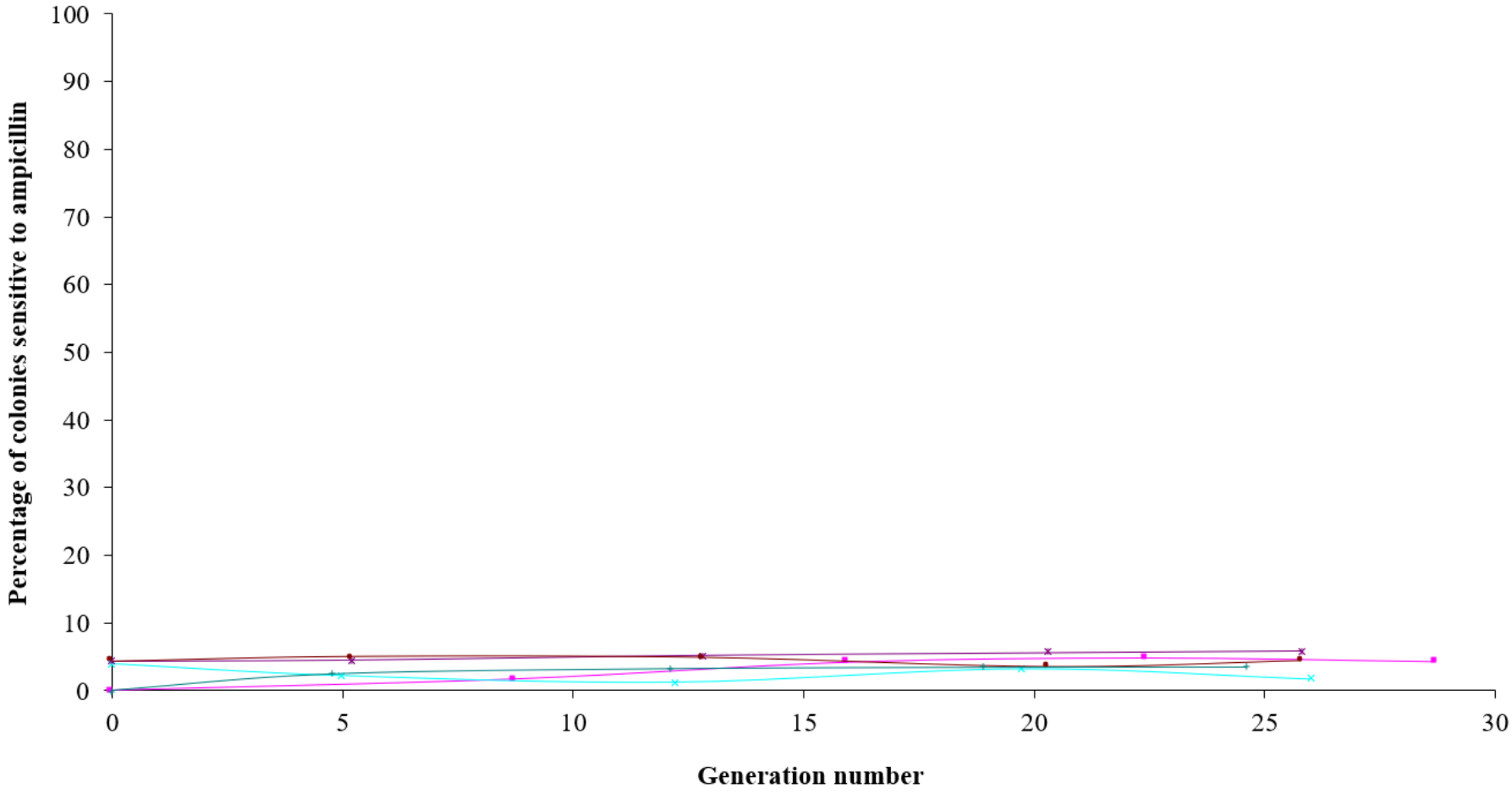


Fig. 5.4: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG4, plotted using a large scale for percentage sensitivity for comparison**

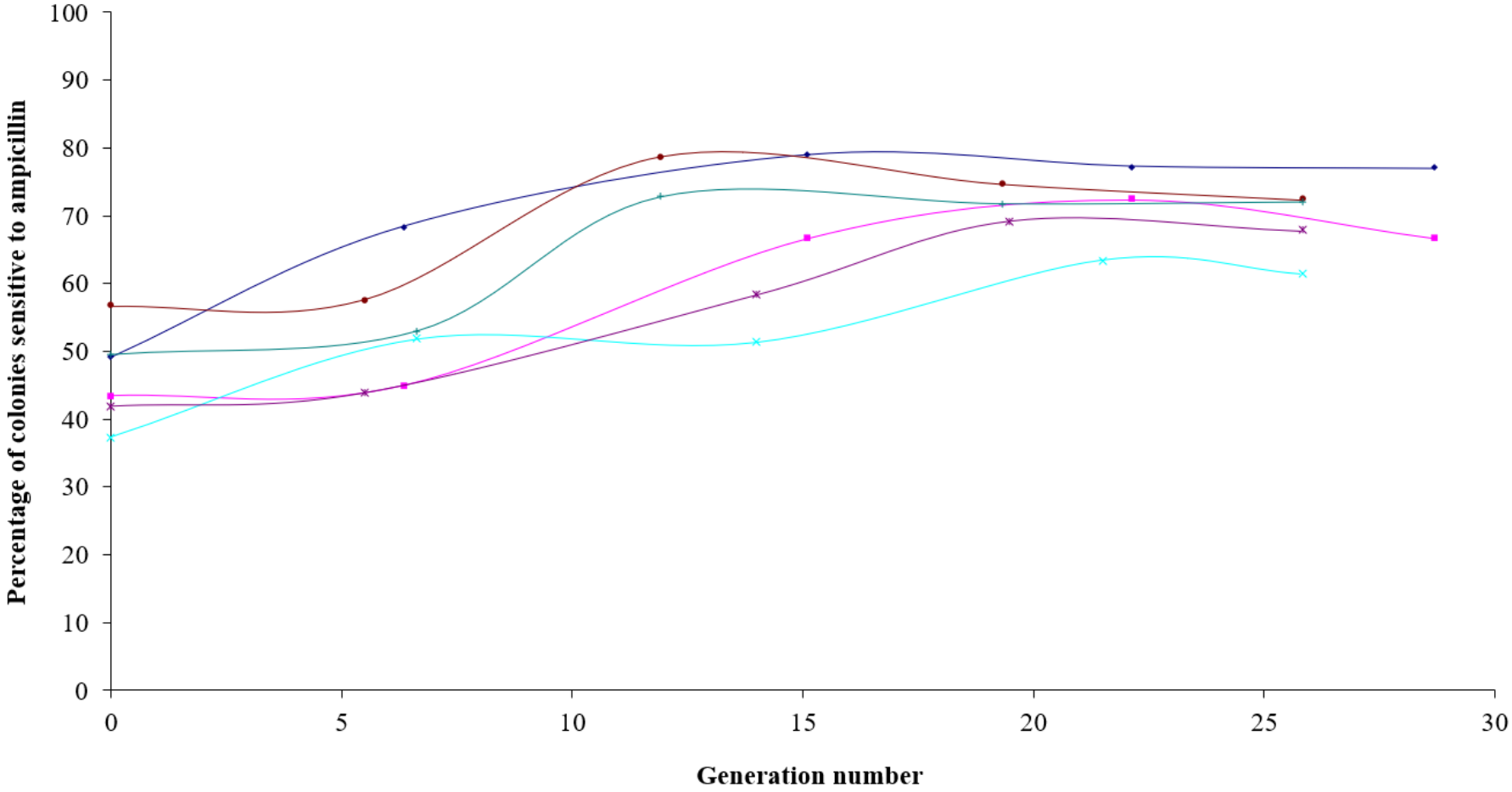


Fig. 5.5: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG04, plotted using a large scale for percentage sensitivity for comparison**

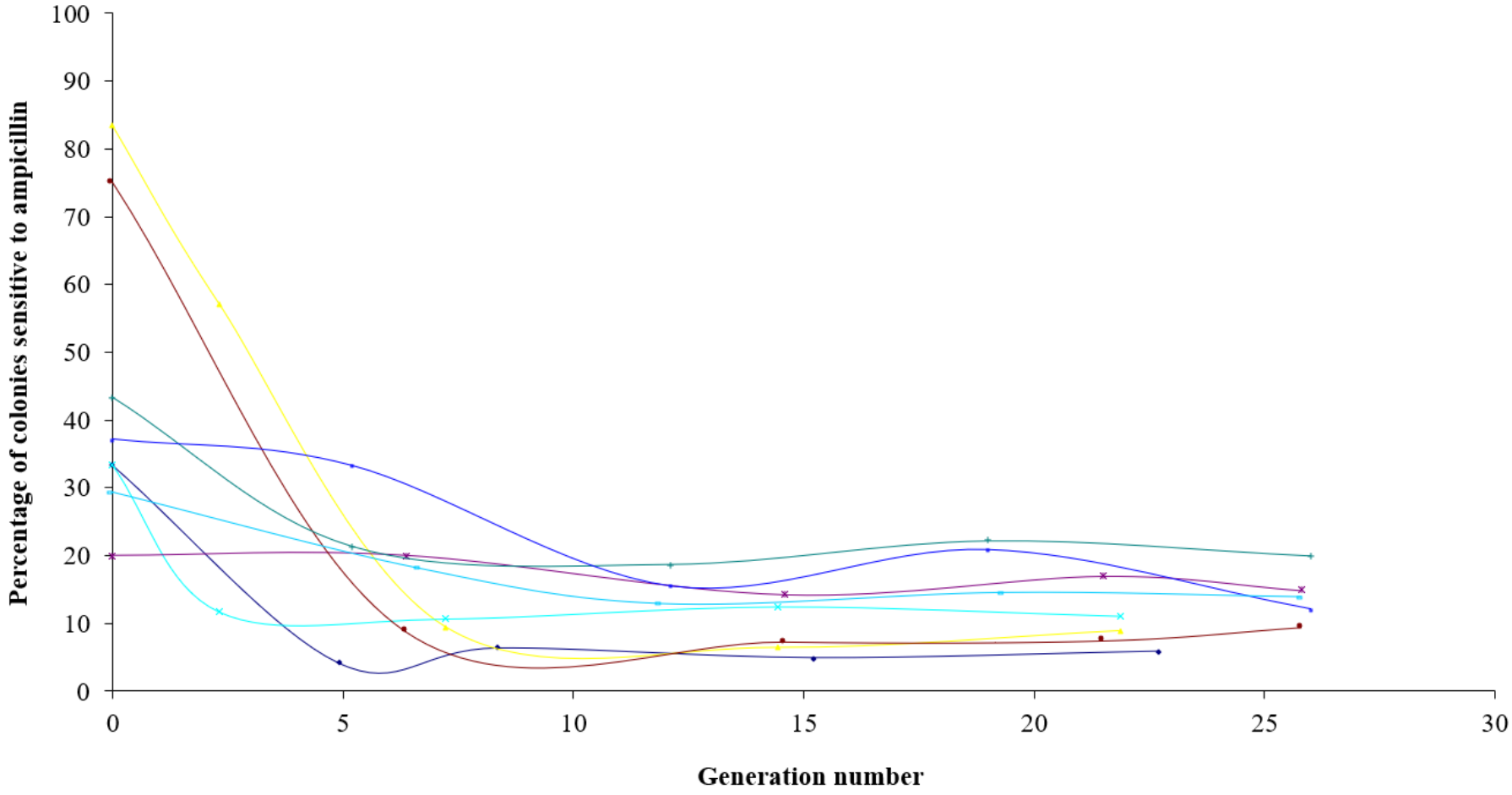


Fig. 5.6: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pOG4.003, plotted using a large scale for percentage sensitivity for comparison**

Figs. 5.1 and 5.2 show the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pALA1029. Three of the cultures showed no sensitivity to ampicillin over the five days of sampling. In the cultures where sensitivity to ampicillin was observed, the maximum percentage of sensitive colonies was approximately 3%. There is variation between the percentage sensitivities observed in these experiments; this is shown very clearly in Fig. 5.1 where it appears that the cultures do not form an equilibrium between plasmid-free and plasmid-containing cells. This is due to the fact that on average, only 100 colonies are replica plated in these experiments. A difference of one sensitive colony will lead to a difference of 1% in the sensitivity of the culture, which is clearly visible when using a small scale such as that seen in Fig. 5.1. Fig. 5.2, which shows the same results but using a larger scale for percentage sensitivity, shows that the variation in the percentage sensitivities of the cultures examined is minimal, a theory supported by the calculation of mean percentage sensitivity of these cultures with 95% confidence limits, the result of which is shown in Fig. 5.14.

Figs. 5.3 and 5.4 show the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pOG4. All cultures showed sensitivity to ampicillin at some point over the course of the five-day experiment. This sensitivity varied between 1% and 6%, excluding the first results taken at time 0 where the culture was not in steady-state. Fig. 5.3 shows variation in the results, suggesting steady-state was not reached in these cultures, but this is again due to the low sensitivity of the experiment when only around 100 colonies are replica plated. Fig. 5.4 shows the same results on a graph with a larger scale for percentage sensitivity. This minimises the variation seen between results, and the calculation of mean percentage sensitivity with 95% confidence limits shown in Fig. 5.14 confirms that this variation is insignificant. It can be observed that there is a difference between the results obtained for experiments with pALA1029 and pOG4; cultures containing pOG4 show an overall higher level of percentage sensitivity that cultures containing pALA1029. The statistical difference between these results is presented in Fig. 5.14.

Fig. 5.5 shows the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pOG04. The cultures all showed a high sensitivity to ampicillin over the course of the experiment. Excluding the results obtained at time 0, when the cultures were not in steady-state, the sensitivity ranged between 44% and 79%. This is

a large range of sensitivity between experiments, but can be explained by small differences between individual chemostat experiments such as temperature, pH, concentration of antibiotics and strength of media (section 7.4.6). It appears that the cultures do not reach steady-state until later in the experiment; at the time that the third sample was taken. After this point there is little variation between the percentage sensitivities of the samples taken from each experiment. The difference between the results obtained for plasmids pALA1029 and pOG4, and those obtained for pOG04 is large. Plasmid pOG04 has a much increased overall percentage sensitivity to ampicillin. The statistical significance of these results is presented in Fig. 5.14.

Fig. 5.5 shows the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pOG4.003. Interestingly in these experiments, the first sample taken generally shows a much higher percentage sensitivity to ampicillin than the results obtained over the course of the experiment. This clearly shows that the culture requires time to form a steady-state, in some cases it is not until the third sample that the culture shows stability. Disregarding the first and second samples because of the lag in forming steady-state, the sensitivity of the cultures ranged from 5% to 22%. Again this variation can be explained by differences in culture conditions within the chemostat (section 7.4.6). The results from these experiments suggest that the sensitivity of cultures containing pOG4.003 is higher than that observed in cultures containing pALA1029 or pOG4, but lower than cultures containing pOG04. The statistical significance of these results is shown in Fig. 5.14, where an overall percentage sensitivity to ampicillin of a culture containing pOG4.003 is presented.

5.3.2 The percentage sensitivity to chloramphenicol of individual steady-state chemostat cultures of *E. coli* C2110 containing pKO1029, pKO4, pKO04 or pKO4.003.

The results obtained from chemostat experiments of *E. coli* C2110 containing the four chloramphenicol resistance-conferring plasmids are shown in Tables 5.5 to 5.8 and Figs. 5.7 to 5.13. For the purpose of comparison, four of the graphs produced use the same scale for percentage sensitivity, showing 0 to 100% sensitivity to chloramphenicol (Figs. 5.8, 5.10, 5.11 and 5.13). The other three graphs (Figs. 5.7, 5.9 and 5.12) show the results for plasmids pKO1029, pKO4 and pKO4.003 using a smaller scale for percentage sensitivity, allowing the individual results to be observed more clearly.

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	110	0	0
	23.5	6.78	116	0	0
	42.6	12.3	102	1	0.98
	67.2	19.4	64	0	0
	89.1	25.7	152	0	0
2	0	0	71	0	0
	23.5	6.78	79	0	0
	42.6	12.3	154	0	0
	67.2	19.4	134	0	0
	89.1	25.7	136	0	0
3	0	0	59	0	0
	21.6	6.23	101	0	0
	44.4	12.8	68	0	0
	69.6	20.1	112	0	0
	89.7	25.9	52	0	0
4	0	0	121	0	0
	21.6	6.23	38	0	0
	44.4	12.8	176	1	0.57
	69.6	20.1	80	0	0
	89.7	25.9	48	0	0
5	0	0	30	0	0
	18.5	5.34	159	0	0
	45.0	13.0	94	3	3.19
	69.6	20.1	98	1	1.02
	91.1	26.3	124	0	0
6	0	0	46	0	0
	18.5	5.34	73	0	0
	45.0	13.0	98	0	0
	69.6	20.1	62	0	0
	91.1	26.3	103	0	0
7	0	0	92	0	0
	16.9	4.89	41	0	0
	44.0	12.7	203	2	0.99
	66.9	19.3	115	0	0
	86.3	24.9	76	0	0
8	0	0	51	0	0
	16.9	4.89	86	0	0
	44.0	12.7	142	0	0
	66.9	19.3	49	0	0
	86.3	24.9	36	0	0
9	0	0	47	0	0
	19.4	5.60	82	0	0
	49.2	14.2	132	1	0.76
	73.5	21.2	123	0	0
	91.1	26.3	89	0	0

Table 5.5: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO1029

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	79	1	1.27
	21.0	6.06	136	6	4.41
	45.7	13.2	184	9	4.89
	66.5	19.2	43	2	4.65
	89.4	25.8	161	7	4.35
2	0	0	92	0	0
	19.5	5.63	35	2	5.71
	48.2	13.9	156	6	3.85
	67.2	19.4	71	3	4.23
	89.7	25.9	65	3	4.62
3	0	0	74	2	2.70
	19.5	5.63	41	3	7.32
	48.2	13.9	45	3	6.67
	67.2	19.4	32	2	6.25
	89.7	25.9	52	4	7.69
4	0	0	82	2	2.44
	21.0	6.06	80	2	2.50
	45.7	13.2	99	2	2.02
	66.5	19.2	130	3	2.31
	89.4	25.8	80	2	2.50
5	0	0	37	0	0
	26.3	7.58	30	0	0
	48.2	13.9	53	1	1.89
	77.3	22.3	143	1	0.70
	90.1	26.0	124	1	0.81
6	0	0	44	2	4.55
	26.3	7.58	40	2	5.00
	48.2	13.9	60	2	3.33
	77.3	22.3	108	4	3.70
	90.1	26.0	86	2	2.33
7	0	0	31	1	3.23
	21.5	6.20	33	1	3.03
	48.2	13.9	294	11	3.74
	65.5	18.9	47	2	4.26
	89.7	25.9	45	2	4.44
8	0	0	46	0	0
	17.1	4.93	38	1	2.63
	45.4	13.1	88	3	2.41
	75.9	21.9	68	1	1.47
	89.4	25.8	95	2	2.11

Table 5.6: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	42	20	47.6
	26.3	7.58	30	24	80.0
	48.2	13.9	36	32	88.9
	77.3	22.3	72	56	77.8
2	0	0	42	26	61.9
	26.3	7.58	58	38	65.5
	48.2	13.9	36	24	66.7
	77.3	22.3	32	24	75.0
3	0	0	51	6	11.8
	20.0	5.77	55	32	58.2
	44.4	12.8	53	35	66.0
	65.1	18.8	74	58	78.4
	88.0	25.4	124	89	71.8
4	0	0	37	10	27.0
	20.0	5.77	36	27	75.0
	44.4	12.8	59	47	79.7
	65.1	18.8	164	135	82.3
	88.0	25.4	125	95	76.0
5	0	0	79	13	16.5
	20.9	6.03	45	29	64.4
	45.7	13.2	62	45	72.6
	66.2	19.1	192	156	81.3
	89.7	25.9	202	146	72.3
6	0	0	30	9	30.0
	20.9	6.03	33	22	66.7
	45.7	13.2	34	30	88.2
	66.2	19.1	36	30	83.3
	89.7	25.9	126	96	76.2
7	0	0	76	11	14.5
	17.3	4.98	140	97	69.3
	43.7	12.6	39	33	84.6
	64.1	18.5	36	30	83.3
	86.3	24.9	72	60	83.3
8	0	0	44	12	24.0
	17.3	4.98	36	24	66.7
	43.7	12.6	42	33	78.6
	64.1	18.5	36	27	75.0
	86.3	24.9	42	33	78.6

Table 5.7: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO04

Chemostat experiment number	Experimental time (hours)	Generation number	Total number of colonies	Number of sensitive colonies	Percentage of colonies sensitive to antibiotics
1	0	0	142	21	14.8
	18.5	5.34	75	27	36.0
	45.0	13.0	136	56	41.2
	69.6	20.1	110	36	32.7
	91.1	26.3	115	40	34.8
2	0	0	104	15	14.4
	17.1	4.93	39	15	38.5
	45.4	13.1	287	108	37.6
	75.9	21.9	227	84	37.0
	89.4	25.8	40	15	37.5
3	0	0	70	11	15.7
	18.7	5.41	88	25	28.4
	46.1	13.3	127	31	24.4
	66.2	19.1	112	26	23.2
	92.2	26.6	50	10	20.0
4	0	0	81	10	12.4
	18.0	5.2	36	12	33.3
	47.1	13.6	93	30	32.3
	69.0	19.9	30	10	33.3
	90.4	26.1	169	57	33.7
5	0	0	14	98	14.3
	18.0	5.2	42	12	28.6
	47.1	13.6	111	28	25.2
	69.0	19.9	101	27	26.7
	90.4	26.1	211	59	28.0
6	0	0	83	12	14.5
	21.2	6.12	33	9	23.1
	48.9	14.1	250	60	24.0
	70.0	20.2	342	85	24.9
	91.5	26.4	31	8	25.8
7	0	0	37	5	13.5
	21.2	6.12	50	20	40.0
	48.9	14.1	46	18	39.1
	70.0	20.2	52	20	38.5
	91.5	26.4	128	50	39.1
8	0	0	0	0	n/a
	18.5	5.34	267	81	30.3
	45.0	13.0	33	12	36.4
	69.6	20.1	110	36	32.7
	91.1	26.3	45	16	35.6

Table 5.8: Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4.003

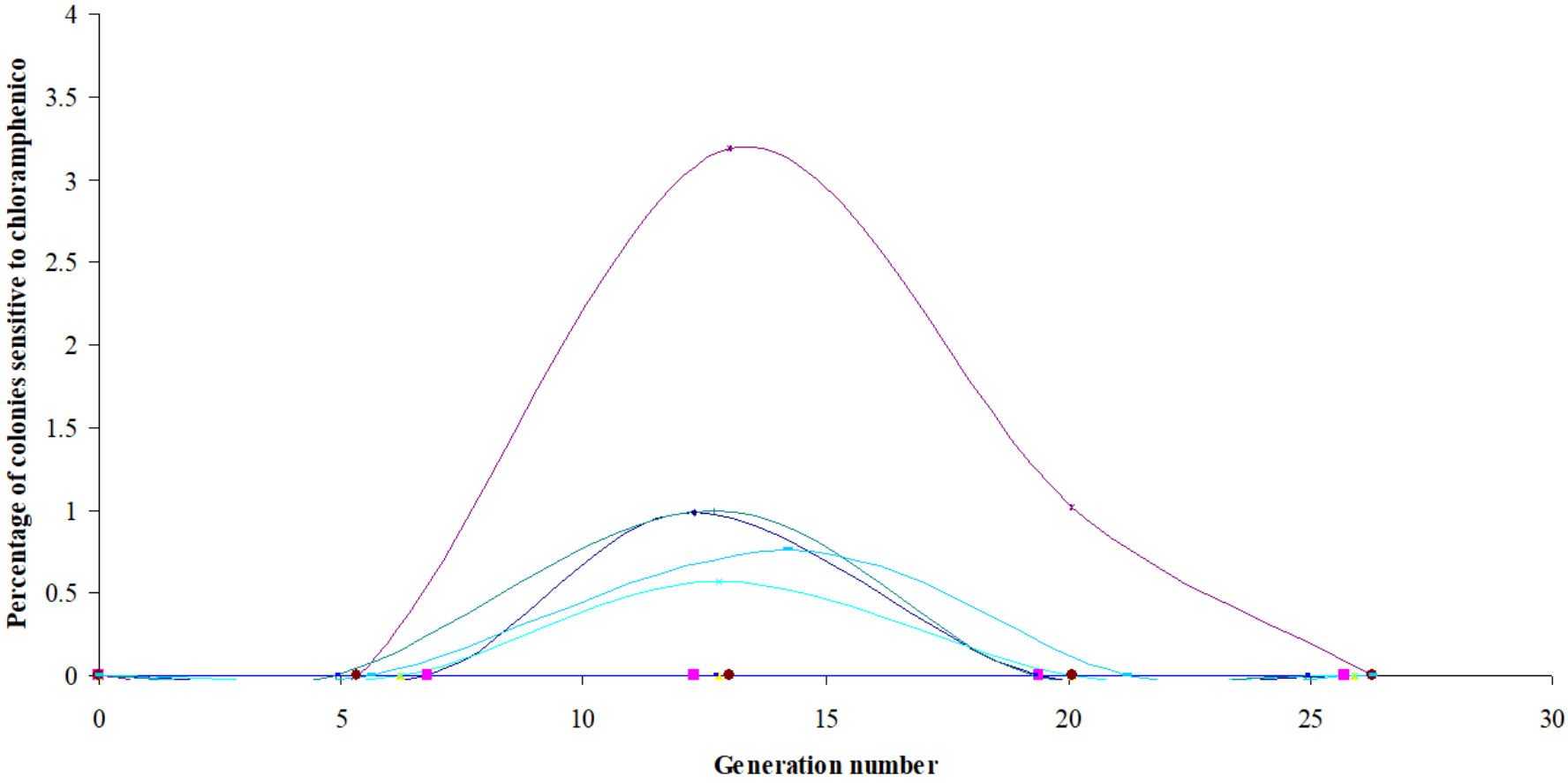


Fig. 5.7: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO1029, plotted using a small scale for percentage sensitivity for clarity**

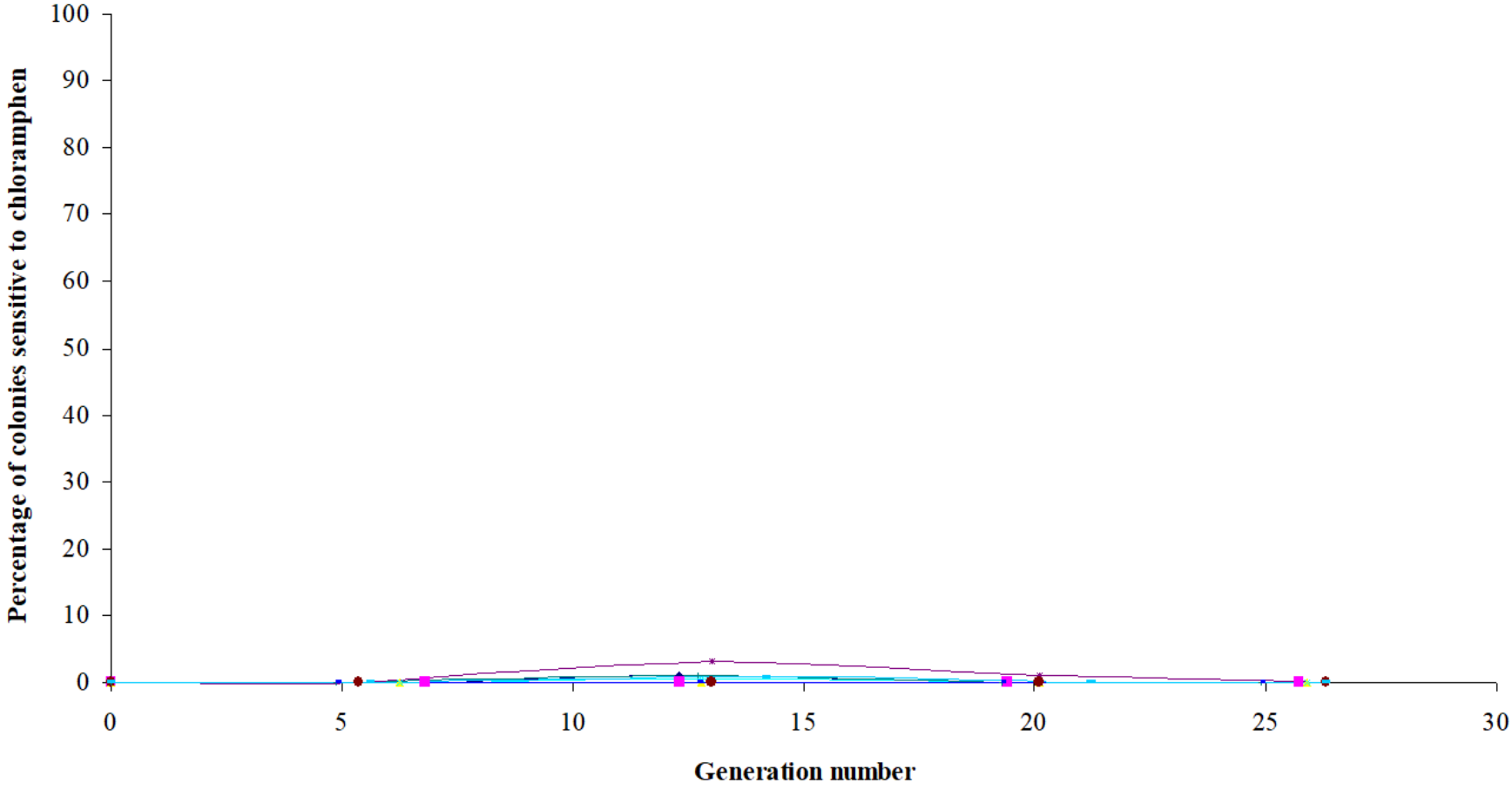


Fig. 5.8: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO1029, plotted using a large scale for percentage sensitivity for comparison**

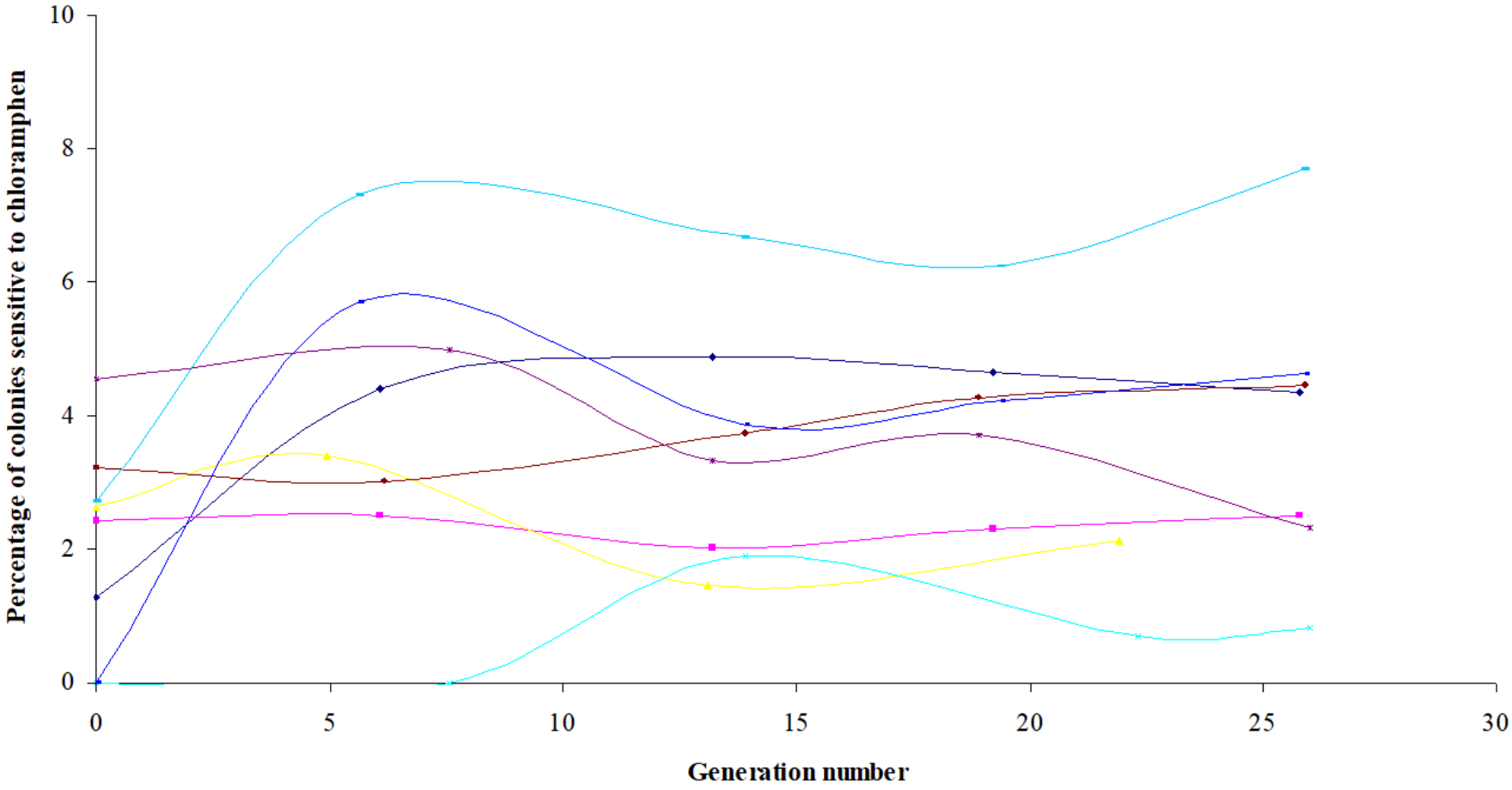


Fig. 5.9: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4, plotted using a small scale for percentage sensitivity for clarity**

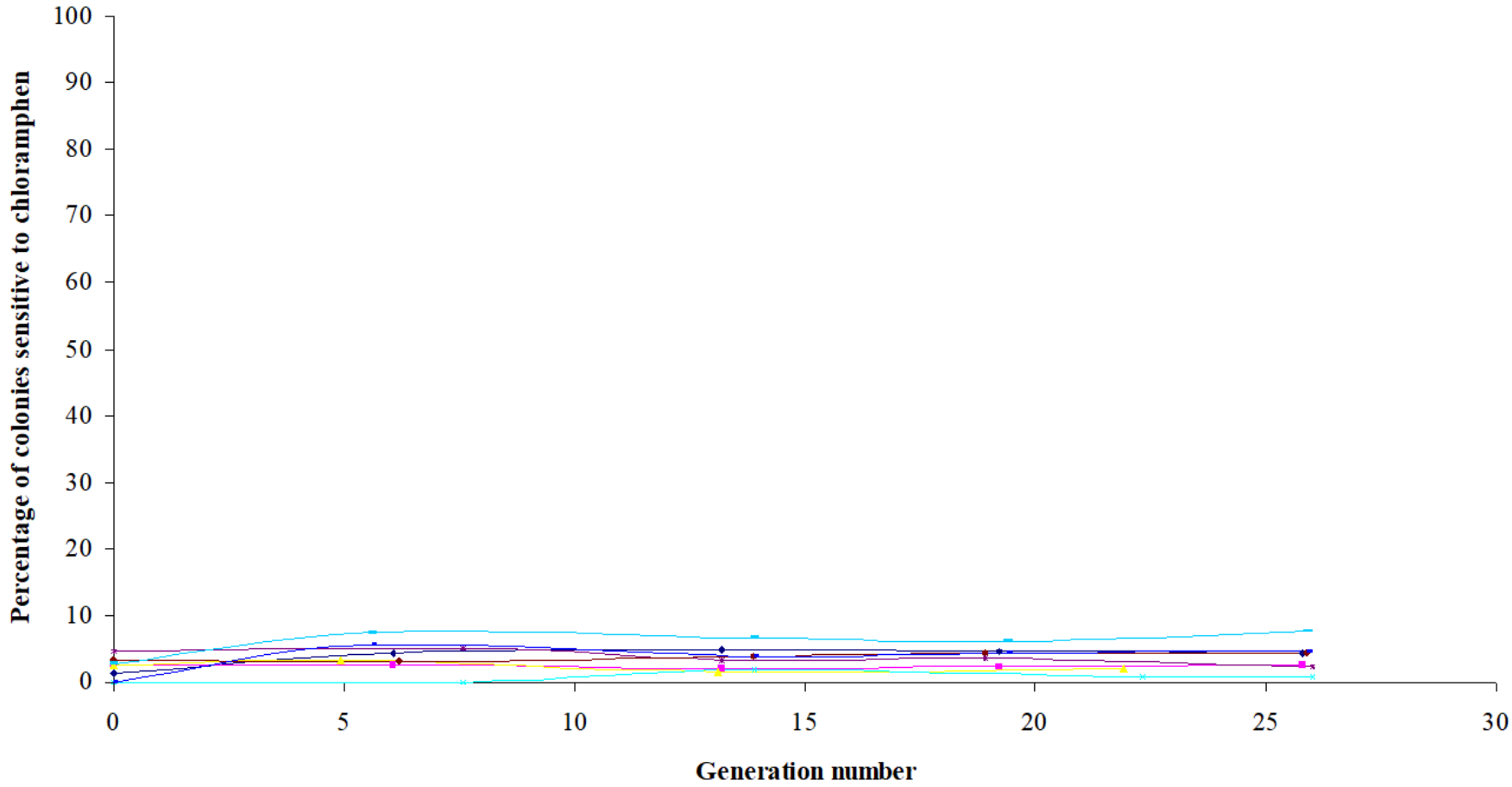


Fig. 5.10: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4, plotted using a large scale for percentage sensitivity for comparison**

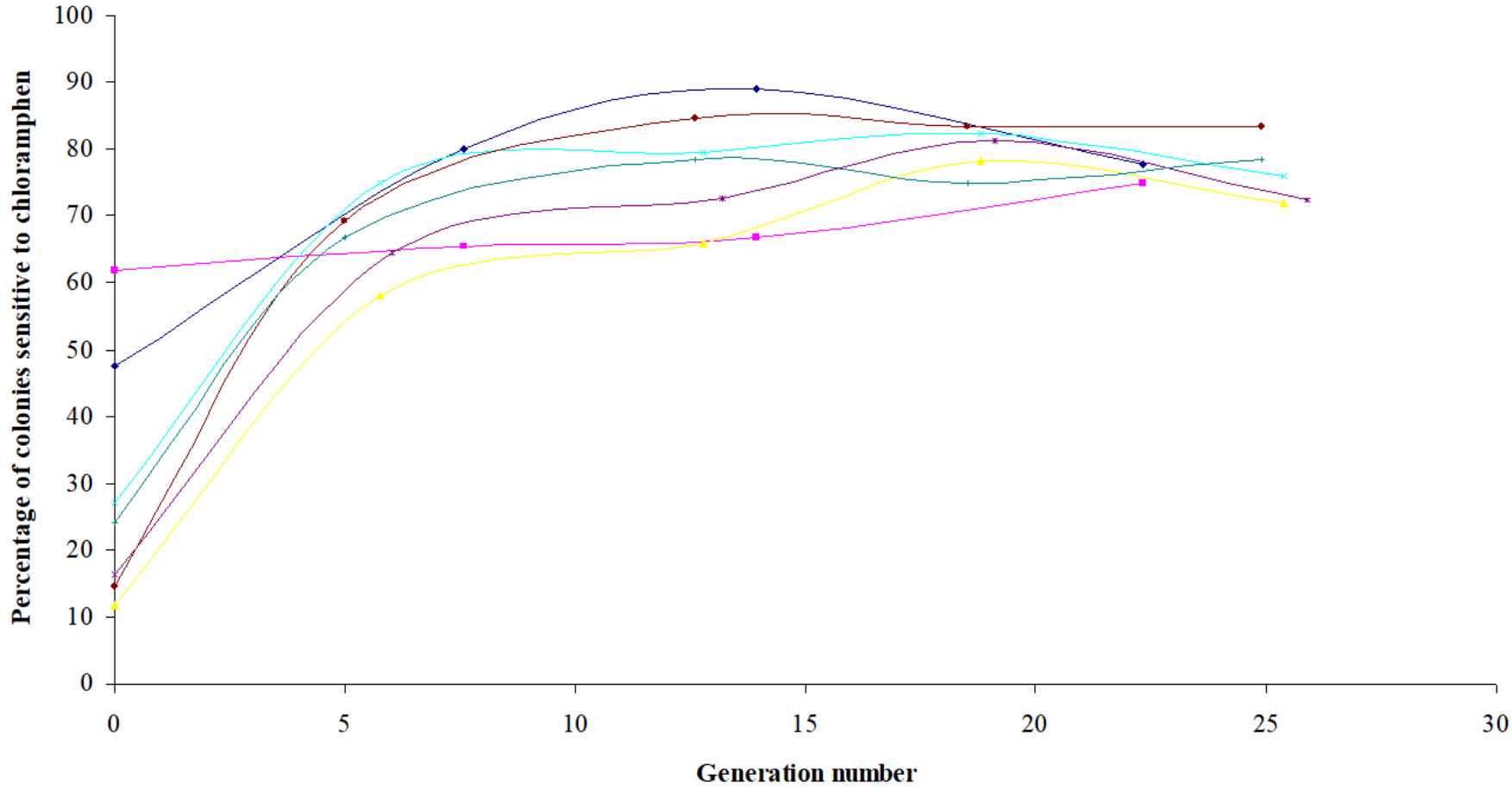


Fig. 5.11: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO04, plotted using a large scale for percentage sensitivity for comparison**

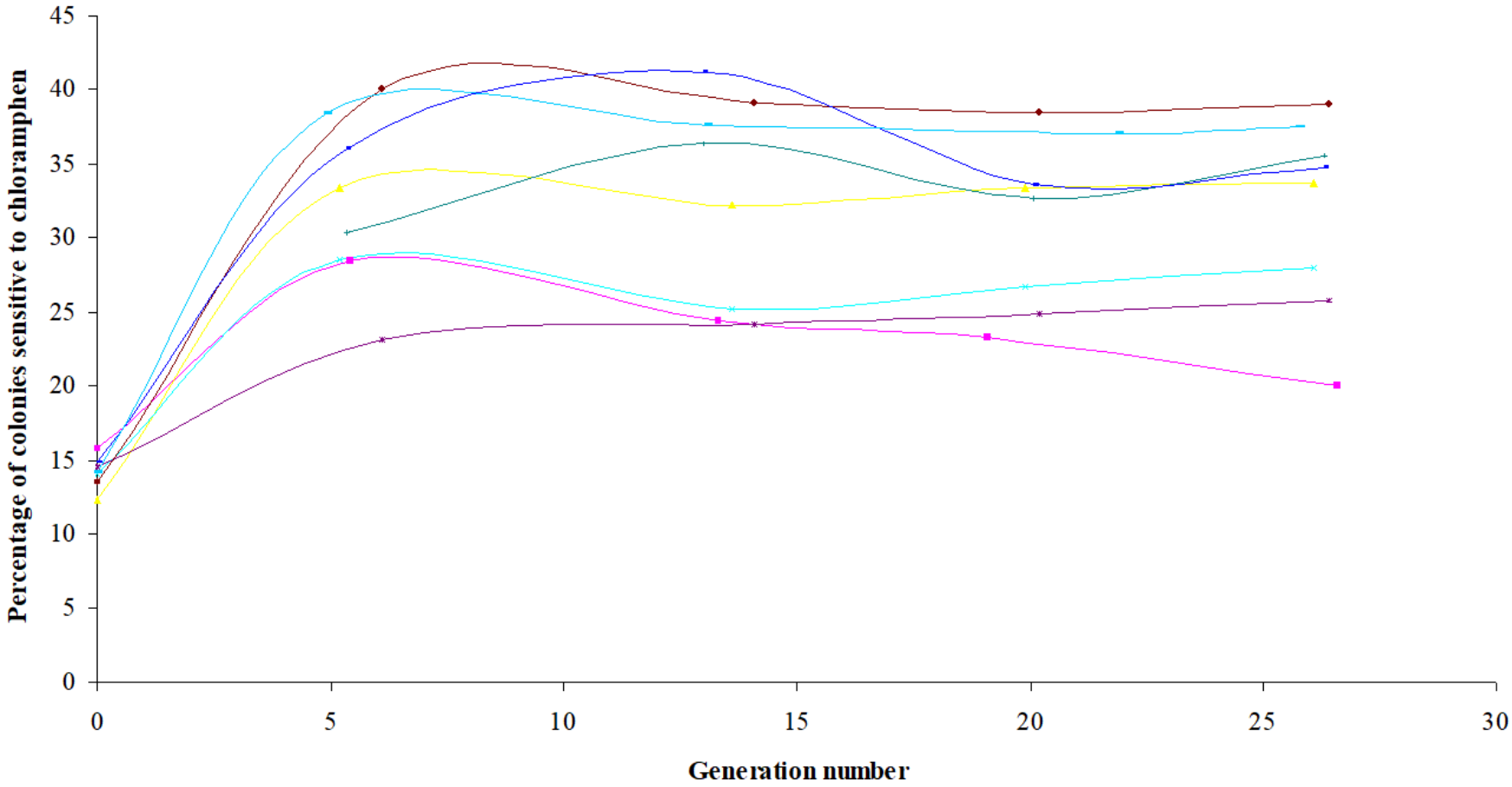


Fig. 5.12: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4.003, plotted using a small scale for percentage sensitivity for clarity**

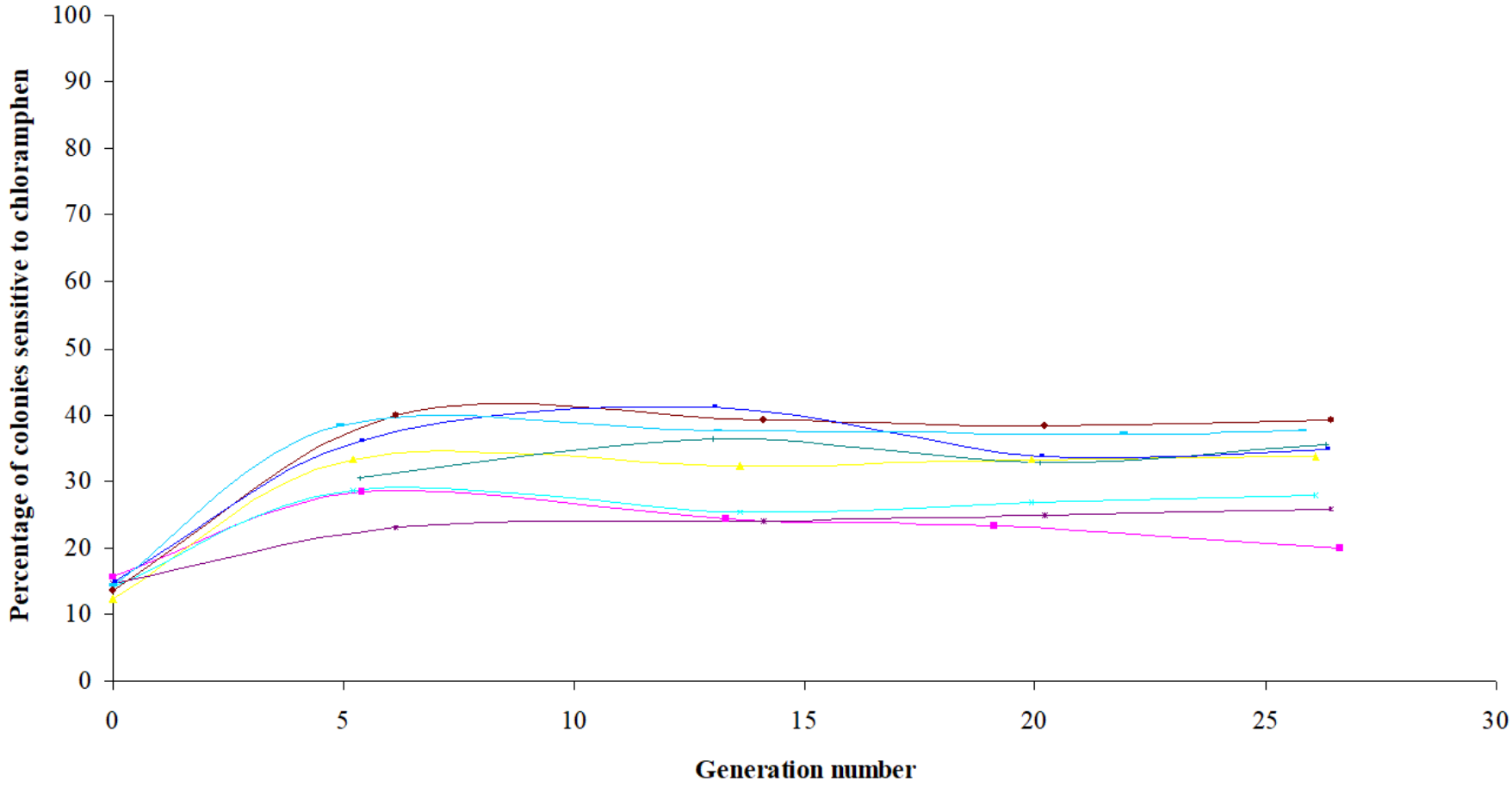


Fig. 5.13: **Results of the individual chemostat experiments for *E. coli* C2110 containing the plasmid pKO4.003, plotted using a large scale for percentage sensitivity for comparison**

Figs. 5.7 and 5.8 show the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pKO1029. Four of the nine cultures showed no sensitivity to chloramphenicol over the course of the whole experiment. The other five cultures showed almost complete resistance to chloramphenicol, with the exception of the third sample, which frequently showed a degree of sensitivity to chloramphenicol. The highest percentage of sensitivity recorded was 3%, although all other percentage sensitivities recorded were below 1%. Consideration of the experimental design will show that for a sensitivity of below 1% to be recorded, over 100 colonies would need to be replica plated. As this was not the case for many of the samples, it is likely that some level of sensitivity was present throughout the course of the experiment, but that it was so low the method of sampling used would have prevented detection. Figs. 5.8 and 5.9 show the result of this limitation in the method, with the appearance of bulges in the graphs suggesting that steady-state had not been reached. The mean percentage sensitivity to chloramphenicol was calculated with 95% confidence limits, and this figure is presented in Fig. 5.14.

Figs. 5.9 and 5.10 show the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pKO4. All of the cultures showed sensitivity to chloramphenicol at some point over the course of the experiment. The highest recorded sensitivity was 8%, while the lowest (discounting the results taken at time 0 when steady-state had not been reached) was just over 1%. Fig. 5.9 shows that most cultures reached some level of equilibrium over the course of the experiment. Variations between individual results taken from the same experiment will be as a result of the limitations in sampling discussed above in relation to plasmid pKO1029. Fig. 5.10 shows that the results obtained for pKO4 suggest a mean percentage sensitivity of approximately 3%, a higher mean than that obtained for plasmid pKO1029. This is confirmed by calculation of the mean percentage sensitivity with 95% confidence limits for both plasmids, the results of which are shown in Fig. 5.14.

Fig. 5.11 shows the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pKO04. The results taken at time 0 suggest a relatively low level of sensitivity to chloramphenicol of the cultures, however by the second sample the sensitivity of the cultures has dramatically increased. This clearly shows the evolution of the culture into a steady-state equilibrium between plasmid-free and plasmid-

containing cells. Discounting the results taken at time 0 for the reason described above, the percentage sensitivities varied between 58% and 89%. This variation can be accounted for by differences in the culture conditions, the most important of which may be the concentration of antibiotics within the culture vessel (section 7.4.6). The mean percentage sensitivity of these cultures with 95% confidence limits is presented in Fig. 5.14, but it can be clearly seen that the percentage sensitivity of these cultures is significantly higher than those obtained for cultures containing pKO1029 and pKO4.

Figs. 5.12 and 5.13 show the results for all the steady-state chemostat experiments for *E. coli* C2110 containing pKO4.003. Similar to the results obtained for pKO04, the percentage sensitivity obtained from the samples taken at time 0 is lower than those obtained from samples at other points during the experiment. This shows that steady-state conditions take time to form within the chemostat. By the second sample however the cultures appear to have reached equilibrium, and after this point there is little variation between the percentage sensitivity results obtained from each experiment. Discounting the results obtained at time 0, the highest percentage sensitivity recorded is 41%, while the lowest percentage sensitivity is 20%. This is different range of sensitivities to those recorded from experiments containing pKO1029, pKO4 and pKO04, suggesting that the mean percentage sensitivity of cultures containing pKO4.003 is significantly different to the means of the other cultures. This can be observed by comparing Fig. 5.13 to Figs. 5.8, 5.10 and 5.11, and is shown graphically in Fig. 5.14.

5.3.3 The mean percentage sensitivity to antibiotics of cultures containing each of the plasmids studied

The mean percentage sensitivity of a culture containing each plasmid was calculated by using all of the results for percentage sensitivity obtained from each individual experiment. The only results not included in the calculation were those from samples taken when the culture was not considered to be in steady-state. For cultures containing plasmids pALA1029, pOG4, pKO1029 and pKO4 the results taken at time 0 were not included in the calculation. For cultures containing plasmids pOG04, pOG4.003, pKO04 and pKO4.003 the results obtained from the first two samples were not included in the calculation.

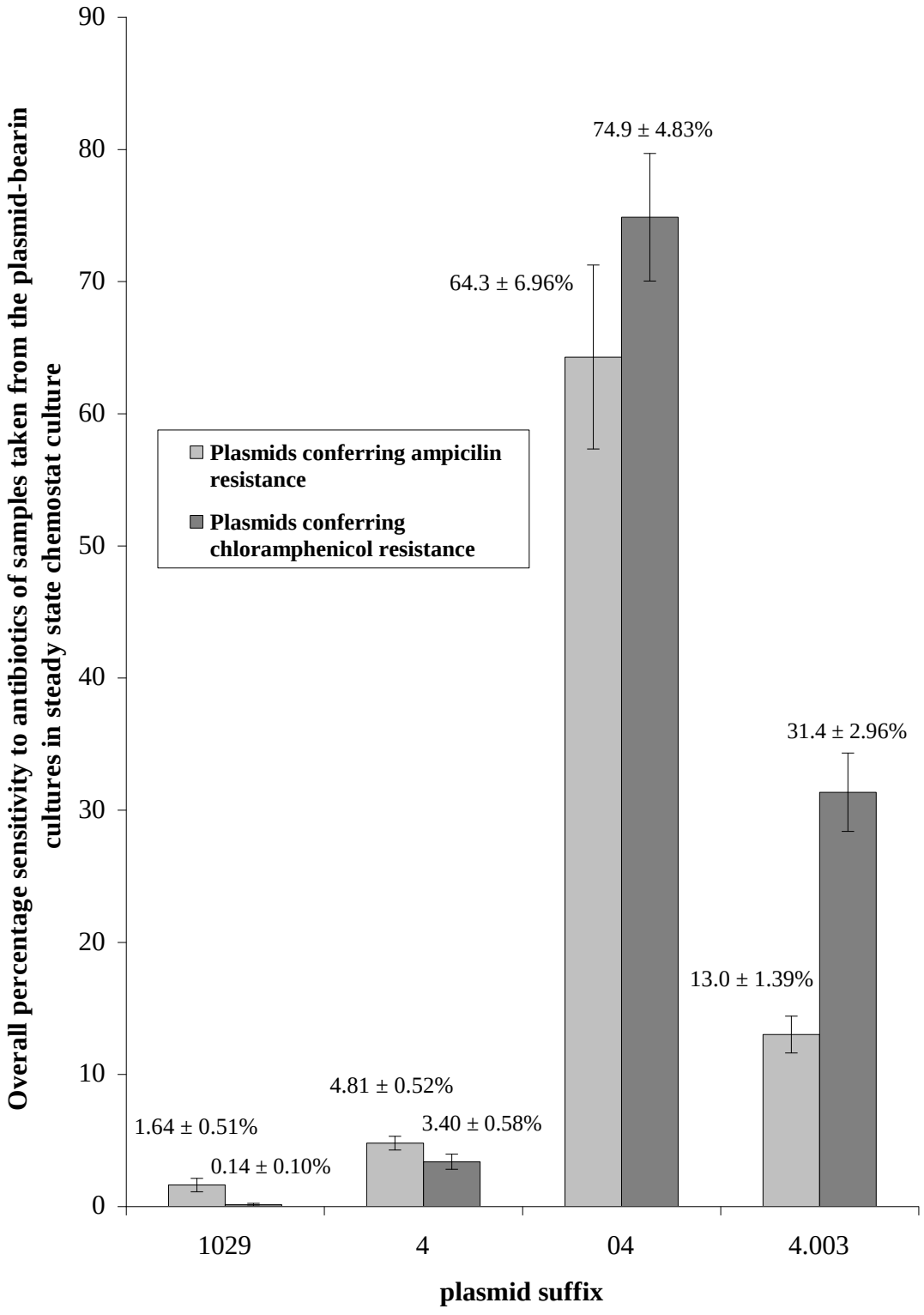


Fig. 5.14: **The mean percentage sensitivity to antibiotics of cultures of *E. coli* C2110 containing each of the plasmids, showing 95% confidence limits.**

The lowest mean percentage sensitivity to antibiotics is that of steady-state chemostat cultures of *E. coli* containing pKO1029 (0.14 ± 0.10%), and although cultures containing pALA1029 also show a low level of sensitivity (1.64 ± 0.51%), the mean is significantly ($P < 0.1\%$) higher than the result for cultures containing pKO1029.

While the mean percentage sensitivity of cultures of *E. coli* C2110 containing either pOG4 or pKO4 are very similar, the result for cultures containing pOG4 (4.81 ± 0.51%) is significantly ($P < 0.1\%$) higher than the result for cultures containing pKO4 (3.40 ± 0.58%).

The mean percentage sensitivity obtained for experiments involving pOG4 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pALA1029. The mean percentage sensitivity obtained for experiments involving pKO4 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pKO1029.

Steady-state chemostat cultures of *E. coli* C2110 containing plasmids pOG4.003 or pKO4.003 show the next highest percentage sensitivity to antibiotics, although the mean percentage sensitivity of cultures containing pOG4.003 (13.0 ± 1.39%) is significantly ($P < 0.1\%$) lower than the mean percentage sensitivity of cultures containing pKO4.003 (31.4 ± 2.96%).

The mean percentage sensitivity obtained for experiments involving pOG4.003 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pOG4. The mean percentage sensitivity obtained for experiments involving pKO4.003 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pKO4.

Finally, steady-state chemostat cultures of *E. coli* C2110 containing either pOG04 or pKO04 show very high percentage sensitivities to antibiotics. Large variation between the percentage sensitivities obtained during the individual experiments has led to large 95% confidence limits, but nevertheless the mean percentage sensitivity of cultures containing pKO04 (74.9 ± 4.83%) is significantly higher ($5\% > P > 2\%$) than the mean percentage sensitivity of cultures containing pOG04 (64.3 ± 6.96%).

The mean percentage sensitivity obtained for experiments involving pOG04 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pOG4.003. The mean percentage sensitivity obtained for experiments involving pKO04 is significantly ($P < 0.1\%$) higher than the mean percentage sensitivity obtained for experiments involving pKO4.003.

The plasmids were checked for structural validity at the end of each experiment (section 2.13). All digests showed identical banding patterns, suggesting that no large structural changes had occurred over the course of the experiments.

5.4 Discussion

5.4.1 The stability of plasmids pALA1029, pOG4, pOG04, pOG4.003, pKO1029, pKO4, pKO04 and pKO4.003 in steady-state chemostat culture.

Steady-state continuous cultures of *E. coli* C2110 containing pALA1029 or pKO1029 show a low mean percentage sensitivity to antibiotics (Fig. 5.14). It was predicted that this would be the case, as the plasmids contain the effective stability system P7*par* (section 1.10.1) and therefore few plasmid-free cells are produced.

Steady-state continuous cultures of *E. coli* C2110 containing pOG4 or pKO4 show slightly higher percentage sensitivities than cultures containing pALA1029 or pKO1029 (Fig. 5.14). These were the expected results, as the *ctl* system (section 1.10.2) present in pOG4 and pKO4 is not as effective at promoting stability as the P7*par* system present in pALA1029 and pKO1029.

Steady-state continuous cultures of *E. coli* C2110 containing pOG4.003 or pKO4.003 show the next highest percentage sensitivities (Fig. 5.14), suggesting that plasmid-free cells are regularly produced at cell division. These plasmids also contain the *ctl* stability system, but the O_B3 binding site is non-functional (section 1.10.3), preventing effective partitioning. As a result these plasmids show significantly ($P < 0.1\%$) higher instability than plasmids pOG4 and pKO4.

Finally, steady-state continuous cultures of *E. coli* C2110 containing pOG04 or pKO04

show a very high level of sensitivity, with a mean percentage sensitivity of approximately 70% in both cases (Fig. 5.14). This indicates that, during cell division, a very high number of cells produced are plasmid-free. These plasmids do not contain a stability system and therefore partition randomly into daughter cells (section 1.10.3).

5.4.2 Mathematical analysis of the results obtained from steady-state chemostat culture

Modelling of the behaviour in batch culture of the plasmids being studied has been previously discussed (section 4.4.3) in relation to the variables R (plasmid loss rate) and $d\mu$ (difference in growth rate of the plasmid-containing and plasmid-free cells). As the plasmids have not been altered between the batch culture experiments and the chemostat culture experiments, the values of R for the plasmids should not have altered.

In contrast $d\mu$ will be dramatically different. In batch culture, the $d\mu$ of all the plasmids studied was positive, as plasmid-free cells had a growth advantage over plasmid-bearing cells because of the reduction in plasmid burden. In the chemostat experiments, however, the plasmid-free cells are now at a serious disadvantage because antibiotics are present within the culture medium.

A study into the stability of a yeast plasmid vector under chemostat conditions highlighted an interesting effect, where the plasmid appeared beneficial to the host strain (Walmsley *et al*., 1983). This would be the case for a culture grown in the presence of an antibiotic where a plasmid conferred resistance to that antibiotic. This situation would best be described as $d\mu < 0$ and $|d\mu| >> R$; in other words, the difference in growth rate between plasmid-free and plasmid-bearing cells is now the most significant factor when considering the cause of plasmid instability in these cultures.

Cooper *et al*. (1987) predicted that, under these conditions in chemostat culture, an equilibrium will form between plasmid-bearing and plasmid-free cells, if R is sufficiently small. This situation was modelled by Cooper *et al*. (1987) and is reproduced graphically in Fig. 5.15.

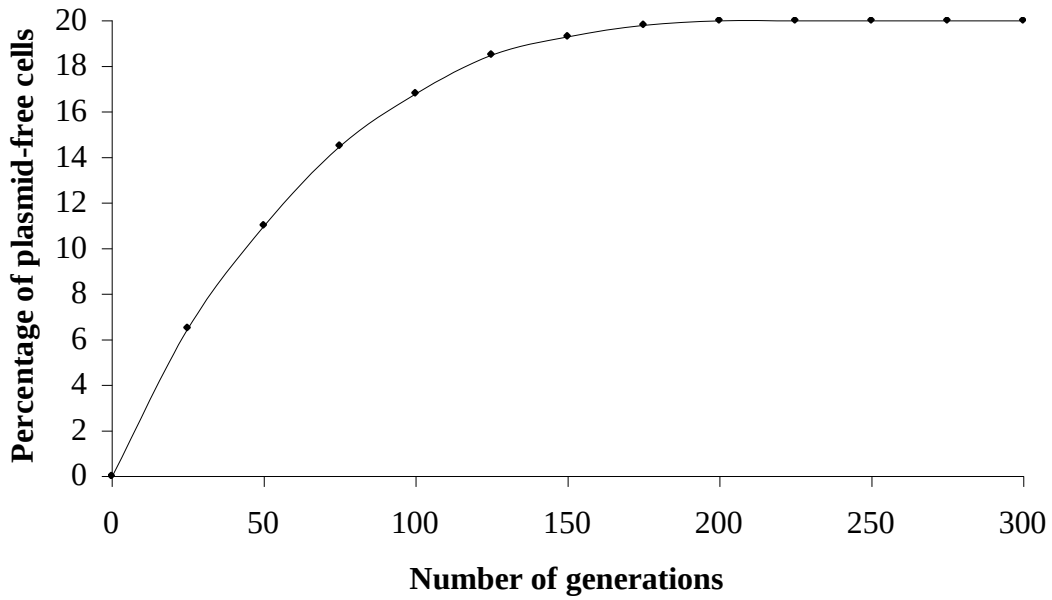


Fig. 5.15: The mathematical model illustrating the increase in plasmid-free cells over generations of growth in a situation described as $d\mu < 0$ and $|d\mu| >> R$ (Cooper *et al*., 1987)

In order for equilibria to form, the rate of plasmid-free cell production must equal the rate of plasmid-free cell loss caused by the action of antibiotics. The percentage sensitivity at which the equilibrium forms is dependent on the value of *R* of the plasmid. The equilibria can form at different percentage sensitivities because the proportion of plasmid-free cells removed by antibiotic action remains constant regardless of the number of plasmid-free cells present in the culture (Cooper *et al*., 1987).

How can any plasmid-free and therefore antibiotic-sensitive cells survive in the presence of antibiotics? Theoretically, any plasmid-free cells exposed to ampicillin should be killed almost instantly, and as exposure to chloramphenicol prevents cell growth, cell death should soon follow.

It is clear from the results obtained that plasmid-free cells do survive in the chemostat; the significant factor to this survival is the constant throughput of cells in the chemostat. The residence time within the chemostat used in this study is 5 hours, which means that all cells and media within the vessel will only be present for five hours at the most. A

certain length of time is required before antibiotics can kill a cell, and cell division could occur during this time, increasing the number of plasmid-free cells in the vessel. Transfer of antibiotic resistance proteins from plasmid-containing mother cells to plasmid-free daughter cells will ensure also their survival for a short time. If during this time a sample is taken, the plasmid-free cells will be plated onto antibiotic-free media, ensuring their survival. Therefore, only a proportion of the plasmid-free cell population present at any one time is removed from the culture by antibiotic action, leading to the observed apparent survival of plasmid-free cells.

The formation of equilibrium shown in Fig. 5.15 will only form if plasmid-free cells are being produced at a low frequency. However, if *R* is very large, then the antibiotics cannot kill plasmid-free cells at the same rate at which they are being produced. As a result, an equilibrium between plasmid-free and plasmid-bearing cells will not form; in fact, the culture will tend towards becoming entirely plasmid-free (Cooper *et al*., 1987). If this is observed in the experiments carried out in this section of work, it would indicate that plasmid destabilisation might be beneficial in clearing a chronic infection. It would also assist in determining the level of destabilisation required for this situation to develop.

Figs. 5.1 and 5.2 illustrate the formation of equilibria in cultures containing pALA1029. There is little variation between the samples at steady state, and the percentage sensitivity of the culture at equilibrium is low. This suggests that the value of *R* of this plasmid is too low to cause clearing of the culture.

Plasmid pOG4 is known to have a higher value of *R* than plasmid pALA1029 (section 4.4.2). Samples taken from the culture containing pOG4 do show a higher percentage sensitivity to antibiotics than samples taken from the culture containing pALA1029. However, it is clear that equilibria still form between plasmid-free and plasmid-bearing cells within these cultures (Figs. 5.3 and 5.4), as there is almost no variation between the percentage sensitivity of the samples taken. Therefore the *R*-value of this plasmid is still too low to cause a clearing of the chemostat culture.

Similar results to those described above can be seen in Fig. 5.7 and 5.8 regarding plasmid pKO1029 and in Figs. 5.9 and 5.10 for plasmid pKO4. For both plasmid-containing cultures equilibria are quickly formed and stably maintained.
On the other hand, steady-state chemostat culture experiments involving pOG04 (Fig. 5.5) and pKO04 (Fig. 5.11), and to a lesser extent pOG4.003 (Fig. 5.6) and pKO4.003 (Figs. 5.12 and 5.13), show a large variation in percentage sensitivities between the samples. In contrast to experiments involving pALA1029, pKO1029, pOG4 and pKO4, the percentage sensitivities do not remain constant from the second sample onwards, suggesting that if equilibria are forming they take a longer time to become stable.

This may suggest that plasmids pOG04, pKO04, pOG4.003 and pKO4.003 have large enough values of *R* to prevent formation of equilibrium within the chemostat cultures. If this is the case then it may suggest that the use of plasmid destabilisation is capable of curing chronic infections.

An important point to raise here is that the only 'destabilised' plasmids are pOG4.003 and pKO4.003. Plasmids pOG04 and pKO04 do not contain a stability system at all. For some reason, these plasmids appear to have become much more unstable in chemostat culture than was expected from the batch culture results. This unexpected result will be discussed further in the following sections.

5.4.3 Comparison of the stability of the plasmids in cultures under steady-state chemostat conditions with the plasmids in cultures under batch culture conditions

It must be made clear that the results obtained from the chemostat culture experiments cannot be directly compared to those obtained from the batch culture experiments. The batch culture results are interpreted to provide a percentage plasmid loss per generation for each plasmid studied. In contrast, the chemostat culture results are expressed as a percentage sensitivity of the population at steady state; no further calculations are carried out on the data. This is because steady-state chemostat cultures should not show any variation in percentage sensitivity over time, and so a percentage plasmid loss per generation could not be calculated. However, comparisons between the two types of

data must be made, and therefore the percentages of sensitive cells within the chemostat cultures are used as an indication of the level of plasmid stability.

Steady-state chemostat cultures of *E. coli* C2110 containing pOG1029 or pKO1029 show low mean percentage sensitivities to antibiotics (Fig. 5.14), suggesting that these plasmids are stable under chemostat culture conditions. As the percentage plasmid loss per generation for these plasmids under batch conditions is also low (Fig. 4.2), this suggests that the different culture methods have had no impact on the stability of the plasmid.

Steady-state chemostat cultures of *E. coli* C2110 containing pOG4 or pKO4 show a higher mean percentage sensitivity than cultures containing plasmids pALA1029 or pKO1029 (Fig. 5.14) suggesting that pOG4 and pKO4 have slightly lower stabilities than plasmids pALA1029 and pKO1029. The same suggestion was made in chapter four, based on the results obtained from the batch culture experiments on these two plasmids (Fig. 4.2). This would suggest that the different culture methods do not affect the stability of these plasmids.

In steady-state chemostat culture, plasmids pOG04 and pKO04 show a very high level of sensitivity to antibiotics (Fig. 5.14), suggesting that the plasmids are extremely unstable under these conditions. In fact, plasmids pOG04 and pKO04 show the highest instabilities of all the plasmids studied in chemostat culture. On the other hand, in the batch culture experiments, plasmids pOG04 and pKO04 showed a lower level of stability than plasmids pOG4 and pKO4, but a higher level of stability than plasmids pOG4.003 and pKO4.003 (Fig. 4.2). Hence it appears that the stabilities of the plasmids pOG04 and pKO04 have changed as a result of the different culture conditions.

Steady-state chemostat cultures of *E. coli* C2110 containing plasmid pOG4.003 or pKO4.003 show higher percentage sensitivities to antibiotics than cultures containing pOG4 or pKO4 (Fig. 5.14). This suggests that both pOG4.003 and pKO4.003 show higher instability in chemostat culture than pOG4 and pKO4. The percentage plasmid loss per generation for plasmids pOG4.003 and pKO4.003 calculated from batch culture are also higher than the percentage plasmid loss per generation for plasmids pOG4 and

pKO4, suggesting that the different methods of culture have not affected the stabilities of these plasmids.

In conclusion, the stabilities of the plasmids pALA1029, pKO1029, pOG4, pKO4, pOG4.003 and pKO4.003 determined in batch culture do not appear to have been dramatically affected by the change in culture method to steady-state chemostat culture. In contrast, the stabilities of the plasmids pOG04 and pKO04 appear to have changed dramatically as a result of the different culture methods.

5.4.4 Explanations as to why pOG04 and pKO04 are more unstable in chemostat culture than batch culture

While the high instability of plasmids pOG04 and pKO04 in chemostat culture (Fig. 5.14) may highlight the possibilities for the action of plasmid destabilisation in clearing a chronic infection, it still remains that this was a highly unexpected result. In batch culture experiments these plasmids were relatively stable, certainly more stable than plasmids pOG4.003 and pKO4.003 (Fig. 4.2). Some of the possible reasons for the change in stability of plasmids pOG04 and pKO04 are described below.

5.4.4.1 The difference in growth rate between batch culture and chemostat culture

The most significant difference between the batch culture and chemostat culture experiments is the growth limitation caused by chemostat culture. In batch culture experiments, cells grew at their maximum growth rate, which for *E. coli* means a cell can divide every 20 minutes (Michelsen *et al*., 2003). Under the chemostat culture conditions used in this study, cell division occurred approximately every 3½ hours. This was due to nutrient limitation caused by a restriction on the flow of fresh medium, and also the use of half-strength LB broth (section 2.3.1).

A reduced growth rate can cause significant physiological changes within bacterial cells, such as changes in cell size and cell mass, an alteration in the number of chromosomes per cell, the DNA to RNA ratios, the number of ribosomes within the cell and changes in protein production (Flickinger & Rouse, 1993). Any of these changes could influence plasmid partition during cell division. It has also been observed that the

metabolic burden of a plasmid on the host cell is larger at lower dilution rates (Ganusov & Brilkov, 2002).

One of the earliest studies on plasmid instability in chemostat culture was to examine how cells carrying the R-factor (R+) survived when grown in a mixed culture with cells lacking this factor (R-). In most growth conditions R+ cells survived well, except under phosphorus limitation where R- cells appeared to compete more effectively. However, the R+ cells were not completely lost from the culture, a small proportion were stably maintained (Melling *et al*., 1977). Alternatively, the small, low copy number plasmid pHSG415 showed increased stability under phosphorus limitation, as well as under potassium and nitrogen limitation. For this plasmid, inheritance became more unstable under sulphate-limited conditions (Caulcott *et al*., 1987). These studies both show that nutrient limitation in chemostat culture can affect the way in which plasmids are maintained. Interestingly, one study has shown that supplementation of the growth media with additional amino acids can actually decrease plasmid stability (Shu & Shuler, 1992).

The pH of the growth media can also affect the stability of plasmids. A study on the plasmids of *Lactococcus lactis* subsp. lactis highlighted that plasmid stability at lower or higher than optimal pH significantly increased the number of plasmid-free cells produced (Béal *et al*., 1998). While the pH within a chemostat culture is controlled, the pH within a batch culture is not, and the difference in pH between the two experiments could be affecting plasmid stability.

All of the possibilities described above should, in theory, affect all the plasmid-bearing strains equally. However, plasmids pOG04 and pKO04 do not contain a stability system, unlike all the other plasmids examined within this study, and therefore it is possible that nutrient limitation within the chemostat will affect these two plasmids differently. This may be the cause of the increased instability, however additional work would need to be carried out to determine the limiting factor within the chemostat system used during this study. Suggestions for further work are described in section 5.4.7.

5.4.4.2 The addition of antibiotics to the culture medium.

The addition of an antibiotic to a culture medium will affect the survival of plasmid-free cells in the population, and a reduction in the proportion of plasmid-free cells would be expected to result in an increased apparent stability. The antibiotics used in these studies should have affected all the cultures in this way; yet only the plasmids pOG04 and pKO04 showed a greatly reduced stability.

5.4.4.3 Structural changes to the plasmids during the experiments.

The plasmids were checked for structural validity at the end of each experiment (section 2.13). All digests showed the same banding patterns, and therefore no noticeable structural changes had occurred over the course of the experiments. However, this does not rule out the possibility of small sequence changes. As neither pOG04 nor pKO04 contain a stability system, the decreased stability cannot be attributed to a change in plasmid partitioning. Also, both plasmids show decreased stability, and the chances of the same structural changes that affect the stability happening simultaneously in both plasmids seems highly improbable.

However, structural changes do not necessarily involve mutation of the DNA. Jones *et al*. (1980) observed that during prolonged chemostat experiments, the copy number of the plasmid pDS1109 was reduced five-fold. This led to an increased sensitivity to ampicillin, as a result of the reduction in the amount of ß-lactamase being produced. A reduction in plasmid copy number was also observed by Wrigley-Jones *et al*. (1993) during studies on plasmid pIJ303 of *Streptomyces lividans*. In addition, there was an increase in supercoiled forms of the plasmid throughout the steady-state chemostat experiment, suggesting that initiation of plasmid replication was reduced (Wrigley-Jones *et al*., 1993). Determining whether any of these structural or topological changes had taken place would require further study into the plasmid structure during all stages of the experiments.

5.4.4.4 Plasmids pOG4.003 and pKO4.003 show an increased stability in chemostat culture conditions

Under batch culture conditions, plasmids pOG4.003 and pKO4.003, which have the

faulty partitioning system, produce more plasmid-free cells than plasmids pOG04 and pKO04, which do not contain a partitioning system. Under chemostat culture conditions, however, pOG04 and pKO04 show the higher instability. Until this point discussions have centred on the assumption that, in chemostat culture, the stabilities of plasmids pOG04 and pKO04 are unusually high, but it is possible that the stabilities of plasmids pOG4.003 and pKO4.003 are unusually low.

The faulty partition system in plasmids pOG4.003 and pKO4.003 allows plasmids to pair prior to division, but the separation mechanism does not function properly and as a result the paired plasmids tend to transfer into a single daughter cell, leaving the other cell plasmid free (Williams *et al*., 1998). However, the reduced growth rate in the chemostat will mean that there will be a long time between cell divisions, and this extra time may allow the paired plasmids to separate and partition correctly. Plasmids pOG04 and pKO04 entirely lack a stability system, so that regardless of the length of time between cell division, plasmid partitioning will still be random. This may explain the greater number of plasmid-free cells produced in cultures containing plasmids pOG04 or pKO04.

5.4.5 A comparison of the stabilities of the ampicillin-resistance conferring plasmids with the stabilities of the chloramphenicol-resistance conferring plasmids

So far the discussion has focussed only on the unusual results obtained for plasmids pOG04 and pKO04. This section will focus on the other results obtained, particularly focussing on the comparison between the ampicillin resistance-conferring plasmids and the chloramphenicol resistance-conferring plasmids.

Steady-state chemostat cultures of *E. coli* C2110 containing pALA1029 show a low mean percentage sensitivity to ampicillin, but the chemostat cultures containing pKO1029 show a significantly lower mean percentage sensitivity (Fig. 5.14). In the batch culture experiments, there was no significant difference in percentage plasmids loss per generation between cultures containing pALA1029 and cultures containing pKO1029 (Fig. 4.2).

In the batch culture experiments, cultures containing pOG4 show a significantly higher instability than cultures containing pKO4 (Fig. 4.2). This is also seen in the chemostat culture experiments where cultures containing pOG4 show a higher instability than cultures containing pKO4 (Fig. 5.14). In other words, the relationship between the two plasmids is the same in both batch and chemostat culture.

The stability of plasmid pKO04 in chemostat culture is lower than that of plasmid pOG04 (Fig. 5.14), whereas in batch culture plasmid pKO04 showed a significantly higher stability than plasmid pOG04 (Fig. 4.2). Therefore the relationship between the two plasmids has been reversed between batch and chemostat culture.

Plasmid pOG4.003 showed a lower stability than plasmid pKO4.003 in batch culture (Fig. 4.2), but in chemostat culture, plasmid pKO4.003 is less stable (Fig. 5.14). Again the relationship between the plasmids has been reversed when comparing batch and chemostat data.

In summary, in batch culture, the plasmids that conferred chloramphenicol resistance generally showed significantly higher stabilities than their related plasmids that conferred ampicillin resistance. This is not the case in chemostat culture. Possible reasons for this change are described in the following sections.

5.4.5.1 The effect of plasmid burden on plasmid stability

Section 4.4.4.5 suggested that the possible reason for the increased stability of the chloramphenicol-resistance conferring plasmids in batch culture was due to differences in the expression of ß-lactamase and CAT. The ß-lactamase promoter is strongly constitutive, producing large amounts of protein and therefore causing a large plasmid burden. In contrast, the promoter responsible for CAT expression is weakly constitutive resulting in a lower level of protein production, and is therefore a reduced plasmid burden, which leads to increased plasmid stability.

In the presence of chloramphenicol, the weakly constitutive promoter would become stronger, ensuring that the host cells are protected against the action of chloramphenicol. This would increase the plasmid burden, possibly above that incurred

by the ampicillin-resistance conferring plasmids, as the chloramphenicol-resistance-conferring plasmids are larger and are also producing a truncated ß-lactamase protein.

A difference in plasmid burden will have a direct effect on the value of $d\mu$, the difference in growth rate between plasmid-free and plasmid-bearing cells. The increased plasmid burden will lead to an increase in the value of $d\mu$, meaning that the plasmid-free cells can overwhelm the plasmid-bearing cells because of their proportionally faster growth rate. But, as described in section 5.4.1, $d\mu$ is negative for the cultures studied in this section because of the presence of antibiotics within the culture medium, which means that plasmid-free cells are unable to grow in the chemostat culture.

However, it was discussed in section 5.4.2 how plasmid-free cells could survive in antibiotic containing media, if only for a short time, and the increased $d\mu$ value could allow the plasmid-free cells to divide more frequently before succumbing to the action of the antibiotic. This could lead to higher percentage sensitivities within cultures containing chloramphenicol-resistance-conferring plasmids.

The maintenance energy of a plasmid may not be the only cause of increased burden to the host cell. It has been observed, for example, that a regulatory protein can inhibit transcription of a chromosomal gene, reducing the ability of the host cell to grow and replicate (Helling *et al*., 1981). This may affect the cultures under steady-state chemostat culture conditions, but not under batch culture conditions leading to a reduction in growth rate of the population.

5.4.5.2 The effects of the different modes of action of the antibiotics on plasmid stability

Although the plasmid-free cells produced within the chemostat culture are susceptible to the action of the antibiotics present in the culture medium, the antibiotics function in different ways. Chloramphenicol is a bacteriostatic antibiotic, meaning that plasmid-free cells will stop growing, but will not be destroyed. Therefore any sample taken from these chemostats will have a number of non-growing but viable cells present within the population. When plated onto antibiotic free agar, the antibiotic restriction

will be lifted and the cells will be able to grow again. As a result, a higher level of sensitive cells will be seen after replica plating, leading to a higher observed percentage sensitivity of the culture.

Alternatively, plasmid-free cells produced in the presence of ampicillin will be killed, as ampicillin is a bactericidal antibiotic. While death may not be instant, in the population as a whole the number of plasmid-free cells will be reduced in comparison to cultures in the presence of chloramphenicol. Therefore, when replica plated, a lower number of sensitive cells will be seen, leading to a lower overall percentage sensitivity of the population.

In addition, chloramphenicol acetyl transferase is a cytoplasmic enzyme that will function immediately after translation of the protein (Stratton, 1996). Low concentrations will be required for effective protection of the cell, and as a result plasmid-free cells will probably contain enough enzyme from the parent cell to provide resistance in the short term. On the other hand, ß-lactamase functions within the periplasm. Once made, the enzyme needs to be transported from the cytoplasm, and a signal protein responsible for transportation has to be cleaved before the enzyme becomes functional (Livermore & Williams, 1996). As a result a much higher concentration of enzyme will be required to provide protection, and therefore plasmid-free cells will not inherit as effective a level of protection to assist in preventing the action of the antibiotic. Therefore, plasmid-free cells in the presence of ampicillin will be destroyed more quickly, leading to a decreased overall percentage sensitivity in the population.

Other possible reasons for increased protection of plasmid-free cells in the presence of chloramphenicol include a greater efficiency of CAT over ß-lactamase, a faster breakdown rate of ß-lactamase as compared to CAT, and a decreased production rate of ß-lactamase within chemostat culture due to the reduced nutrient availability.

Variations in the concentration of antibiotics present in the culture medium may also be responsible for producing unusual results. For example, if chloramphenicol were broken down more quickly than ampicillin in the chemostat, then more plasmid-free cells would be able to survive in the presence of the reduced chloramphenicol

concentration. Also, it would be expected that the cultures of cells containing plasmids pALA1029 and pKO1029 would be exposed to a lower concentration of antibiotic than cultures containing plasmids pOG04 and pKO04, because of the difference in the quantity of resistance enzymes produced by the plasmid-bearing cells. Temperature, exposure to light, and aeration could also affect the concentration of antibiotics within the culture vessel. Therefore, the concentration of antibiotics within the chemostat vessel was considered to be a useful parameter to assay and control, as differences between the antibiotic concentrations might help to explain the different levels of stability observed. The experiments carried out in order to determine the antibiotic concentrations within the culture vessel are described in chapter 6.

5.4.5.3 The higher than expected stabilities of plasmids pKO1029 and pKO4.

While sections 5.4.5.1 and 5.4.5.2 may explain the decreased stability of plasmids pKO04 and pKO4.003 in comparison to plasmids pOG04 and pOG4.003, it does not account for the results obtained for plasmids pKO1029 and pKO4. Plasmid pKO4 shows an almost identical stability to plasmid pOG4, while pKO1029 is significantly more stable than pOG1029 in chemostat culture.

One possible reason is that plasmids pKO1029 and pKO4 show an inherent increase in stability due to an increase in copy number, or to a small structural change within the plasmid. Other than this, no explanation for the increased stabilities can be given, as all the hypotheses given in this discussion would suggest that the chloramphenicol-resistance-conferring plasmids should show a higher level of instability in chemostat culture. Further experiments would have to be carried out to explain these results, and examples of possible experiments are given in section 5.4.6.

5.4.6 Experiments that could provide further insights into the causes of the differences in plasmid stability

The main aim of this section of work was to determine the *R* value required to clear a chemostat culture in the presence of antibiotics. It has been suggested that plasmids pOG04, pKO04, pOG4.003 and pKO4.003 may be unstable enough for this to occur, as the formation of equilibrium was unclear (section 5.4.2). The easiest way of testing this

theory would be to run longer-term chemostat cultures of cells containing these plasmids, for example, over a period of a month. This length of time should allow full clearing of the chemostat if it were to occur at all. If clearing did occur, then further experiments using plasmids with different *R* values may help to pinpoint more accurately the level of destabilisation required for clearance to occur.

Several experiments could be carried out to determine whether the differences between the chemostat and batch culture results for plasmids pOG04 and pKO04 are purely due to growth rate and nutrient limitation (section 5.4.4). One would involve taking a single antibiotic resistant colony from a final chemostat sample and using it to run a batch culture experiment. If the stability of the plasmids recorded from these experiments were similar to those recorded in chapter four, then any differences could be ascribed to a change in growth rate, and not to structural changes within the plasmid.

Another possibility would be to run a steady-state chemostat experiment at a very high dilution rate, allowing the cells to grow as close to μ_{max} as possible. This would provide the culture with almost unlimited nutrients and a growth rate similar to that seen in the batch culture experiments. If growth rate were the only cause of this decreased stability, then it would be expected that the order of plasmid instability would return to that seen in the batch culture experiments, with plasmids pOG04 and pKO04 showing a higher stability than plasmids pOG4.003 and pKO4.003. Experiments of this kind are described in chapter seven.

Experiments carried out with other plasmids that do not contain a stability system may prove that the unusually high instability of plasmids pOG04 and pKO04 in chemostat culture is not limited to these plasmids. If this effect can be observed with completely unrelated plasmids, then it would suggest that random plasmid partition is generally ineffective at slow growth rates.

The reason for the higher than expected stabilities of plasmids pKO1029 and pKO4 in chemostat culture remains unknown (section 5.4.5.3). Alternative plasmids could be selected from the stocks created after ligation (section 3.6) and used in both batch and chemostat experiments. If different results were obtained by using different plasmids,

then it is possible that the plasmid stocks currently being used have structural changes that affect their stabilities.

It was suggested in section 4.4.5 that new plasmids could be constructed which contained both a functional ß-lactamase gene and a functional CAT gene, and cultures containing these plasmids could be grown in either ampicillin or chloramphenicol. This would prevent any variation in the results obtained through a change in $d\mu$, as the plasmids would have the same burden when grown in either ampicillin or chloramphenicol. Changing the promoter controlling expression of CAT to the strongly constitutive promoter controlling ß-lactamase production would also reduce variability caused by different plasmid burdens in the presence or absence of antibiotics.

5.5 Conclusion

The aim of this section of work was to determine the level of plasmid instability required to assist in the clearing of a chronic bacterial infection. A steady-state chemostat was used as a model of a clinical infection, and the antibiotic-resistant plasmid-containing cultures were subjected to antibiotic pressure.

The results obtained showed that equilibria between plasmid-free and plasmid-containing cells do form in the chemostat in the presence of antibiotics, confirming the validity of the model proposed by Cooper *et al*. (1987). This was particularly apparent for cultures containing stable plasmids, such as pALA1029, pOG4, pKO1029 and pKO4.

In contrast, cultures containing plasmids that are known to be unstable, such as pOG04, pOG4.003, pKO04 and pKO4.003 appear to form equilibria in the chemostat less readily. This also validates the Cooper *et al*. (1987) model, which states that plasmids with a high value for *R* will not form equilibria, and, given enough time, will eventually lead to a complete clearing of the chemostat. This complete clearing was not observed during the experiments carried out for this study, but the data obtained here would suggest that this would be the case over a longer experimental time.

Many unexpected results were obtained from the steady-state chemostat culture experiments carried out in this section of work. It appears that growth rate has an effect on plasmid stability, supported by the differences in results obtained from batch culture experiments when compared with those obtained from chemostat culture experiments. It also appears that the alteration of the plasmids to confer resistance to chloramphenicol has affected plasmid stability. The reasons for these changes in stability have not been identified at the present time, but if the further work suggested in section 5.4.6 was to be carried out, then these matters may be resolved.

The conclusion that can be drawn from this section is that the destabilisation of plasmids that confer antibiotic resistance would be effective in reducing the problem of antibiotic-resistant bacterial infections, as it has been shown that an increase in plasmid instability leads to a higher percentage of plasmid-free cells within a culture; cells that are now susceptible to antibiotics.

CHAPTER SIX – DETERMINATION OF THE ANTIBIOTIC CONCENTRATIONS IN CHEMOSTAT CULTURE

6.1 The use of ampicillin and chloramphenicol in clinical infections

The introduction to chapter five (section 5.1) states that the chemostat is being used as a simplified model of a clinical infection for this study. Although steady-state chemostat culture has more similarities to an infection than batch culture, there are still many areas in which the model is lacking. For example, no competing microbial cultures are present in the chemostat culture used in this study. There is no equivalent to the host immune system in chemostat culture, which affects the cell growth rate by increasing the temperature or through damage caused by white blood cells. There will be variation in the nutrients provided by the host during the course of the infection. The use of general antimicrobials, such as iodine, will reduce the cell number periodically during an infection. However, one way to improve the chemostat model would be to ensure that the antibiotic concentration in the chemostat is the same as that found at the site of an infection. A literature search was carried out to determine the use of ampicillin and chloramphenicol in clinical situations, and the concentrations of these antibiotics at sites of infection.

An oral preparation of antibiotics is the most commonly used form of medication for bacterial infections. For example, an oral preparation of 2 g of ampicillin gives a peak serum concentration of 109-150 μg ml^{-1}. However, within three hours the concentration of ampicillin in the serum is negligible (Lister & Sander, 1995). A 500 mg oral dose of ampicillin can provide a concentration of 2000 μg ml^{-1} in the urine (Drobot *et al*., 1996), suggesting that oral preparations of antibiotics are very effective for treating urine infections. Intravenous injections are a fast way of ensuring higher doses of antibiotics in the bloodstream, and suggested peak serum concentrations are between 177 and 200 μg ml^{-1} (Nahata *et al*., 1999). This data suggests that a serum concentration of between 100 to 200 μg ml^{-1} is most commonly obtained, and therefore the concentration of ampicillin currently used in the chemostat experiments is slightly low at 100 μg ml^{-1}.

Chloramphenicol is a relatively toxic compound, often causing haematological

disorders, and therefore is not widely used as a therapeutic agent. The recommended level of chloramphenicol in the serum is 10-25 μg ml^{-1}, as a higher concentration increases the occurrence of side effects (Ismail *et al*., 1998). Therefore the chloramphenicol concentration of 20 μg ml^{-1} included in the chemostat media is similar to that seen in a clinical infection.

However, although a concentration of chloramphenicol at 20 μg ml^{-1} and ampicillin at 100 μg ml^{-1} is added to the fresh medium, and this may not reflect the concentration present in the culture vessel during the course of the experiment. It is very likely that the concentration present in the vessel will be lower than the levels described above for several reasons. The increased temperature and aeration present in the chemostat will cause some antibiotic breakdown, but the degradative enzymes coded for by the plasmids will be responsible for a large drop in antibiotic concentration. An accurate way of determining the residual level of the antibiotic in the culture vessel is required, and this is most easily carried out by using high-performance liquid chromatography (Wright *et al*., 1998). High performance liquid chromatography (HPLC) is regularly used to determine antibiotic concentrations as it is a fast, sensitive and accurate technique (Luo *et al*., 1997). Once the residual concentration is determined, a higher concentration of antibiotic can be added to the culture medium to ensure that the steady state concentration of antibiotics is at the correct level to imitate clinical infections, and the effect of this change on the stability of the plasmids can be seen.

6.2 Measurement of ampicillin by high performance liquid chromatography (HPLC)

As ampicillin was used in higher concentrations (100 μg ml^{-1}) than chloramphenicol (20 μg ml^{-1}) within the chemostat, initial experiments were carried out with ampicillin using a system determined by literature searches. The mobile phase was a mixture of 14 parts sodium phosphate buffer (0.05M at pH7) to 5 parts acetonitrile (Klepser *et al*., 1997; Erah *et al*., 1998). The Hypersil ODS 5 μm C-18 column (150 x 4. 6 mm I. D.) (Erah *et al*., 1998) was used for separation of the ampicillin, with a flow rate of 1 ml min^{-1} controlled by a Bromma, 2150 HPLC pump. Detection of the peak was carried out at 235 nm (Klepser *et al*., 1997; Erah *et al*., 1998) using a Perkin Elmer LC-85B spectrophotometer, and the results were collected on a Shimadzu C-R6A

Chromatopac integrator plotter.

6.2.1 Measurement of pure ampicillin dissolved in the mobile phase

In order to assess the suitability of HPLC for the determination of the concentration of ampicillin, an initial experiment was carried out on pure ampicillin. This also allowed for optimisation of the equipment used during the experiments to produce a clearly detectable peak.

Ampicillin was dissolved in the mobile phase to produce final concentrations of 25, 50, 75 and 100 µg ml^{-1}. An example of a peak produced by analysis of ampicillin at a concentration of 100 µg ml^{-1} is shown in Fig. 6.1.

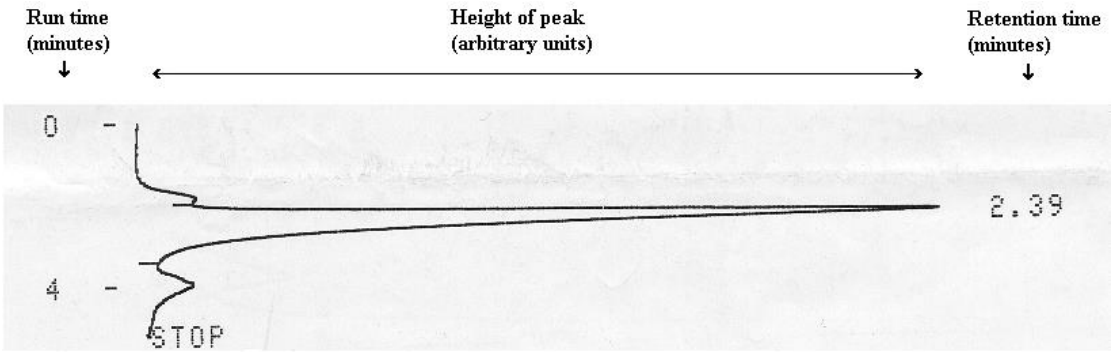


Fig. 6.1: Chromatogram showing the peak recorded from analysis of ampicillin dissolved in the mobile phase to a concentration of 100 µg ml^{-1}

Fig. 6.1 illustrates that ampicillin was detected as a clean peak, apart from a small quantity of degradation products at the base. The retention time of ampicillin under these HPLC conditions averaged at 2.5 minutes (the retention time of the peak seen in Fig. 6.1 is 2.39 minutes). The height of the peak was recorded at the end of the run; the height of the peak seen in Fig. 5.4 is 3109 arbitrary units. Each concentration of ampicillin was analysed by HPLC on three occasions, and an average of the peak height was calculated with 95% confidence limits (section 2.14.1). The results were used to produce the calibration graph shown in Fig. 6.2.

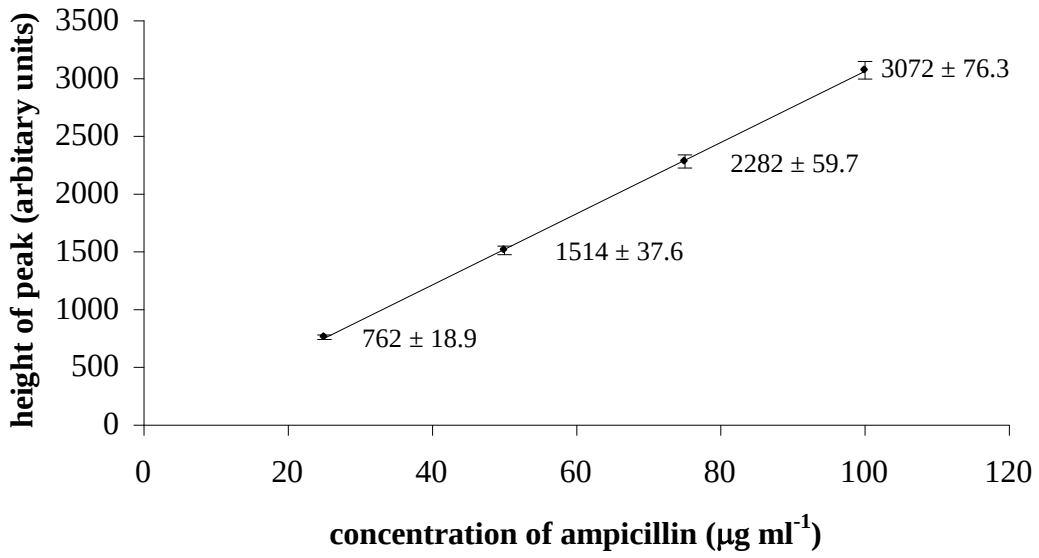


Fig. 6.2: A graph showing the height of the peak detected by HPLC for different concentrations of ampicillin with 95% confidence limits

Fig. 6.2 shows the relationship between the peak heights detected by HPLC and different concentrations of ampicillin dissolved in the mobile phase. The correlation between the height of the peak and the ampicillin concentration is directly proportional and very reproducible; the variation between replicate results for all four concentrations was less than 1%.

6.2.2 Measurement of the concentration of ampicillin in samples taken from chemostat culture

It was clear that measurement of pure ampicillin dissolved in the mobile phase would be easier than measurement of ampicillin in growth medium, which would also contain bacterial cells and degradative enzymes. In order to reduce the effects of contamination, samples taken from the chemostat were preconditioned before HPLC analysis. Immediately after sampling, the culture was placed on ice to reduce the action of enzymes that may have degraded the antibiotics (Schügerl & Seidel, 1998), and then centrifuged at 15,000 **g** for two minutes to remove the bulk of the cells. Finally, the supernatant was passed through a 0.45 μm filter to ensure that any cells remaining in the supernatant were removed (Wright *et al*., 1998). During centrifugation and filtration

the sample was kept cold, and the preconditioned sample was left on ice until analysis by HPLC was carried out. Fig. 6.3 shows a peak recorded from a preconditioned sample of culture medium containing ampicillin under exactly the same HPLC conditions used for detection of ampicillin in the mobile phase.

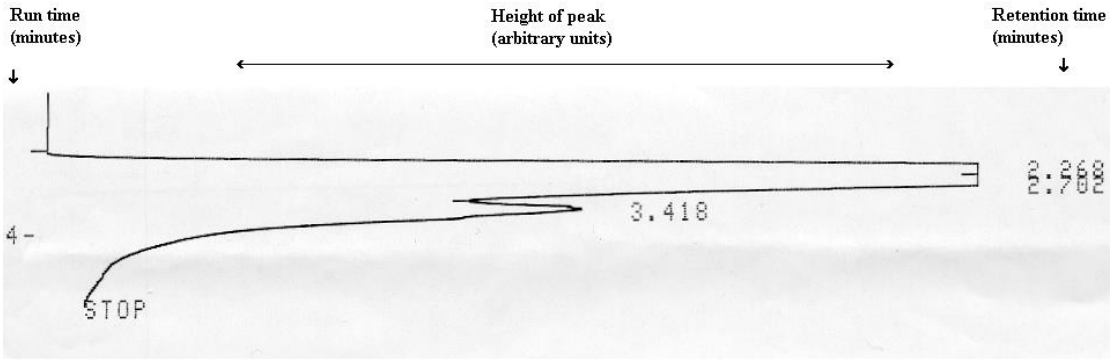


Fig. 6.3: Chromatogram showing the peak recorded from analysis of a preconditioned sample taken from chemostat culture

Analysis by HPLC requires that the compound being studied produces a peak which is separate from any other contaminating compounds. Fig. 6.3 shows that the ampicillin peak cannot be distinguished from components of the culture medium using the current analysis method. The wavelength for detection of the peak was altered in an attempt to separate out the ampicillin peak, however this made no obvious difference to the result. To confirm that the overlapping peaks were not caused by cell or enzyme contamination, a spiked control (sterile half-strength LB broth (2.3.1) containing ampicillin at a concentration of 100 µg ml^{-1}) was analysed by HPLC. The peak produced from this spiked control was the same as that seen in Fig. 6.3.

It was clear at this point that the ampicillin would have to be separated out in order to obtain measurements that can be read using the calibration graph (Fig. 6.2). Klepser *et al*. (1997) extracted ampicillin from complex solutions with acetonitrile after evaporating off any excess water. As acetonitrile was a component in the mobile phase there would be no problems with incompatibility. A fresh spiked control was produced and evaporated in a vacuum to provide a concentrated pellet. The pellet was

redissolved in 500 µl of acetonitrile, and 20 µl of the resuspended solution was run on the HPLC column. The peak recorded from this sample is shown in Fig. 6.4.

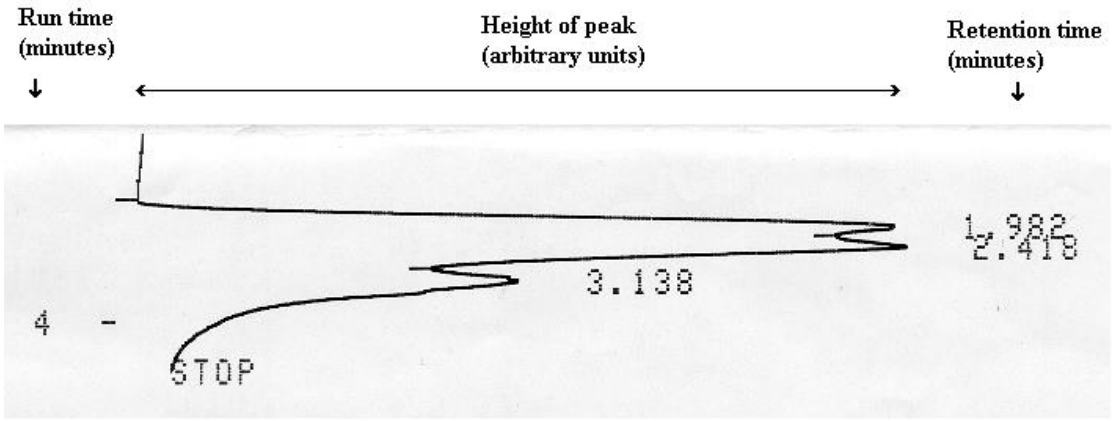


Fig. 6.4: Chromatogram showing the peak recorded from analysis of an acetonitrile extraction from a dried pellet of half strength LB broth containing 100 µg ml^{-1} of ampicillin

Fig. 6.4 shows that acetonitrile extraction did not assist in producing a clear peak as some of the culture medium components also appeared to be soluble in acetonitrile. It can also be seen that the peak heights are lower, suggesting that either a lower proportion of the components have dissolved in the acetonitrile, or the centrifugation step has degraded all of the compounds due to heat damage. It should be appreciated that vacuum centrifugation is carried out at room temperature, and possibly at higher temperatures caused by heat generated by the motor, and therefore that the ampicillin could be degraded during this process, although evaporation of the solvent should cool the sample to some extent. It is possible that the peak appearing at 2.418 minutes is the ampicillin peak, however, this peak is not clear enough to allow for accurate determination of the concentration of the antibiotic.

6.3 Measurement of concentrations of pure chloramphenicol by high performance liquid chromatography (HPLC)

Although measurement of ampicillin in culture medium by HPLC was problematic, an attempt to measure chloramphenicol levels by the same technique was carried out.

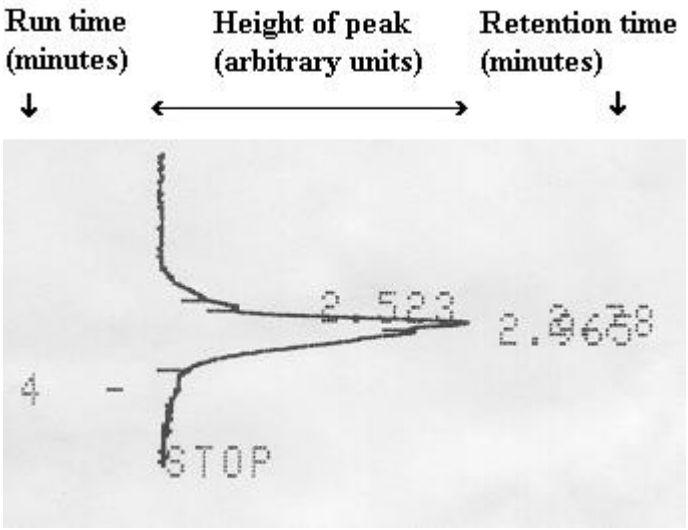


Fig. 6.5: Chromatogram showing the peak recorded from analysis of chloramphenicol dissolved in the mobile phase to a concentration of 20 µg ml^{-1}

Fig. 6.5 shows the peak produced by pure chloramphenicol dissolved in the mobile phase. It can be seen that the peak runs off the column at a very similar time (2.8 to 3 minutes) to that of ampicillin. Because of this, further work was not carried out, as the chloramphenicol peak would also be masked by components of the LB broth, and there would be the further difficulty that the peak produced was significantly smaller, as lower concentrations of chloramphenicol were used in chemostat culture.

6.4 Measurement of concentrations of ampicillin by an agar plate based bioassay

With the conclusion that the HPLC method was flawed, alternative techniques for the measurement of antibiotic concentrations were considered. One alternative method for determining levels of antibiotic in tissues, fluid or media is by the use of microbiological methods. A microbial agar diffusion assay can show the antibiotic concentration by determining the extent of clearing of bacteria in agar plates (Drobot *et al*., 1996). Microbiological methods are time consuming, and often have poor reproducibility and accuracy, but because of the complications of using HPLC to determine concentrations of antibiotic in culture media, a preliminary study was carried

out on the suitability of a bioassay for determination of antibiotic concentrations within the culture medium.

The bioassay was carried out using several variables to determine which method would be the most effective. Pour plates and spread plates were prepared containing either *E. coli* C2110 or *E. coli* DH5α (table 2.1). Four different concentrations of ampicillin were prepared at 100, 75, 50 and 25 μg ml^{-1}, and the antibiotic was added to the cells by addition of a small filter disc soaked in the ampicillin concentration, or by forming a well in the agar and adding 0.5 ml of the prepared stock concentration. A flow chart to show the combinations used is shown in Fig. 6.6.

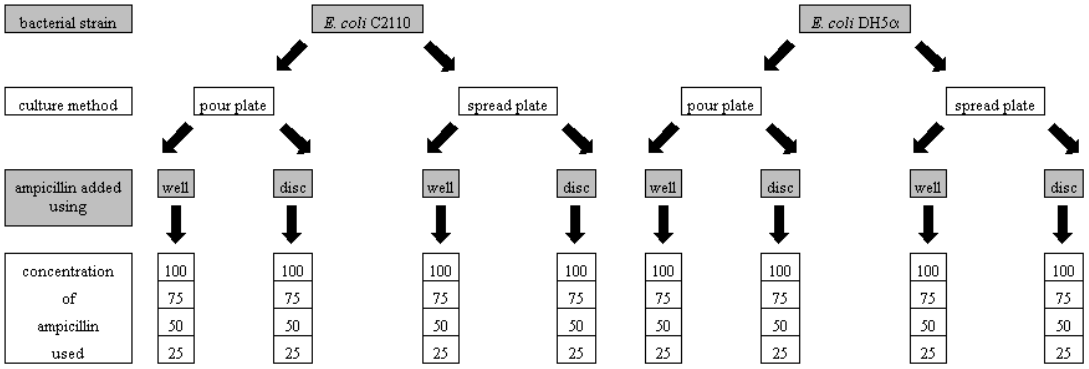


Fig. 6.6: A flow chart to show the combinations of *E. coli* strain, antibiotic concentration and method of supplying antibiotic for the initial bioassay evaluation.

After overnight growth of the cells at 37˚C, measurements of zones of clearing were taken for all of the prepared plates. Pour plates containing *E. coli* C2110 with a well formed in the centre produced the most apparent zones of clearing and the largest variation between the diameter of clearing at the lowest and highest concentrations of ampicillin. Figs. 6.7 and 6.8 show examples of the clearing seen after overnight growth.

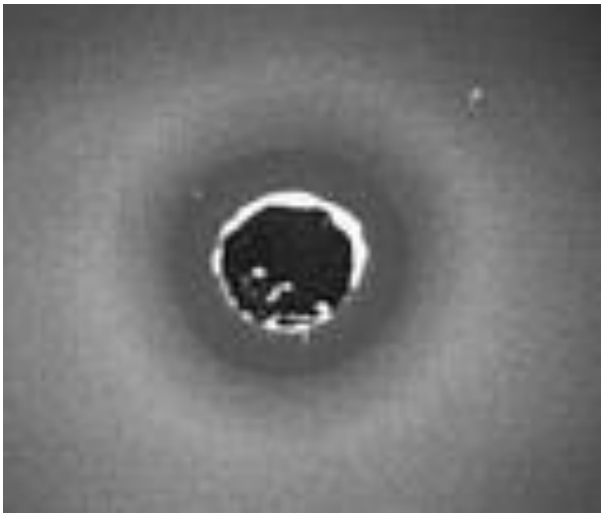


Fig. 6.7: The zones of clearing visible on a pour plate containing *E. coli* C2110 after addition of 0.5 ml ampicillin at a concentration of 100 µg ml^{-1} to a well in the centre

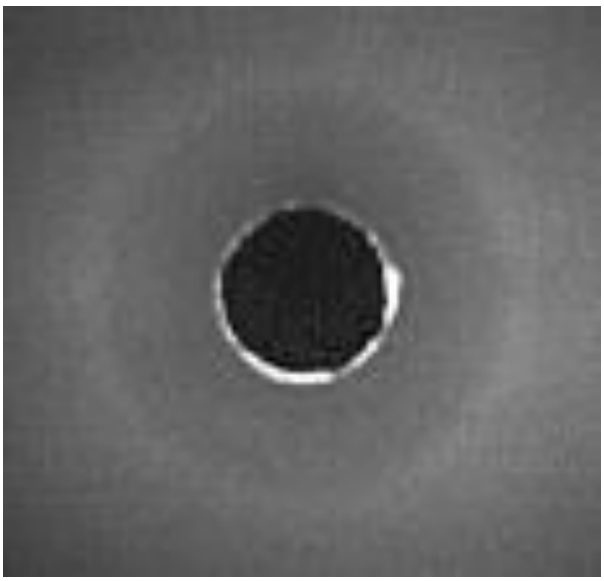


Fig. 6.8 The zones of clearing visible on a pour plate containing *E. coli* C2110 after addition of 0.5 ml ampicillin at a concentration of 50 µg ml^{-1} to a well in the centre

Two zones were observed around the well with the presence of higher concentrations of ampicillin, a completely cleared area showing no bacterial growth and a slightly cleared area showing minimal growth as seen in Fig. 6.7. Lower concentrations of ampicillin

did not cause complete clearing of bacterial cells in any part of the plate as seen in Fig. 6.8. However, two zones were still apparent, one showing slightly less clearing than the other. An average of three replicas was taken in this initial experiment and 95% confidence limits were determined (section 2.14.1). The results are shown in Fig. 6.9.

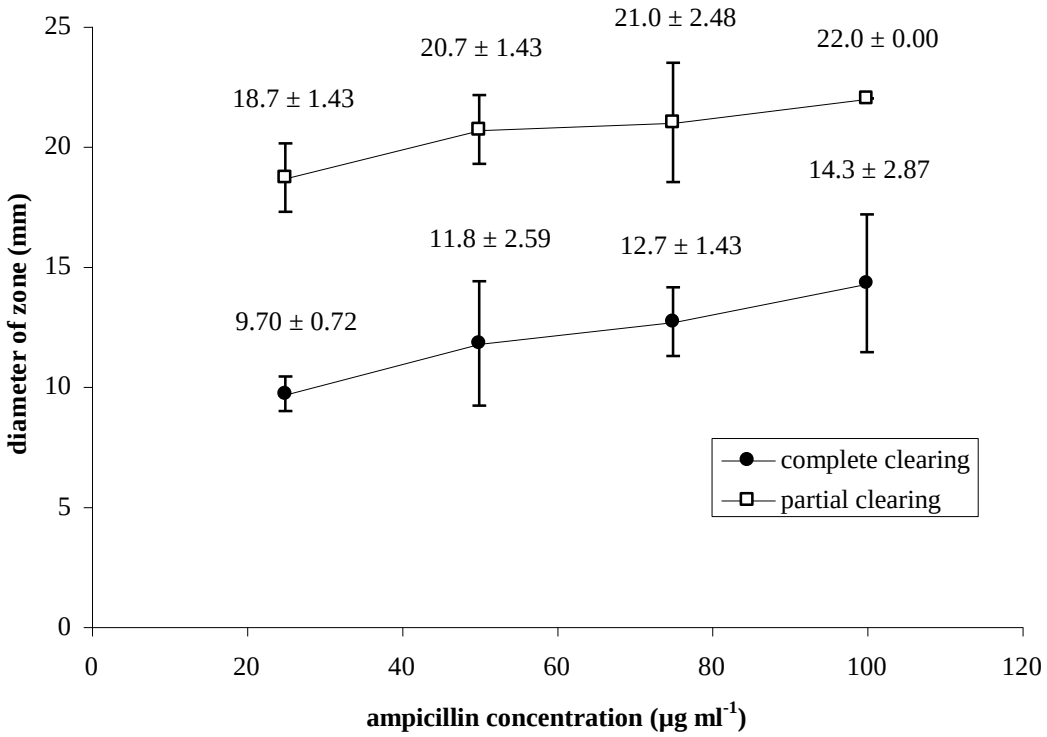


Fig. 6.9: A graph showing the zones of clearing, with 95% confidence limits, observed in pour plates containing *E. coli* C2110 with ampicillin added to a well

There is no significant difference (P > 20%) between each of the increasing ampicillin concentrations, although there is a significant difference (5% > P > 2%) between the zone of clearing with an ampicillin concentration of 25 µg ml^{-1} and 100 µg ml^{-1}. However, this is not a high enough level of sensitivity to be of use in accurately determining the antibiotic concentrations within the chemostat vessel.

6.5 Discussion and conclusion

High performance liquid chromatography is used frequently in the drug production industry to determine the concentration of antibiotics in the cultures. The difference between these systems and the one used in this study is the concentration of the antibiotic. When drugs are being produced on an industrial scale, the concentration is very much higher than used in the chemostat for this study. As a result, the peak recorded from industrial fermenters for ampicillin is far larger and overwhelms any peak produced by growth media.

It therefore follows that further reductions in the concentration of antibiotics in the chemostat experiments in this study would further hamper the efforts to analyse the concentration by HPLC. These reductions would be expected as a result of bacterial enzymatic action within the culture vessel.

While microbiological methods are more time-consuming, they would have been appropriate to use in this study, however the lack of sensitivity prevents them from being helpful for the analysis of low levels of antibiotics.

In conclusion, although measurement of the antibiotic concentration within the chemostat would have assisted in the analysis of results, perfecting the techniques would have taken too long, particularly considering that the results obtained would not have been essential to furthering the main aim of the study.

Further work in this area would involve refining the HPLC technique to allow determination of low concentrations of antibiotics, or repeating the chemostat experiments using a very high concentration of antibiotics in order to ensure that, regardless of the amount of degradation, the antibiotic concentration should remain above the threshold for effective action against plasmid-free cells. This would assist in determining the effect, if any, differing concentrations of antibiotics would have on plasmid stability in a steady-state chemostat culture (section 5.4.5.2).

CHAPTER SEVEN – THE EFFECTS OF PLASMID INSTABILITY ON THE GROWTH RATES OF CULTURES UNDER WASHOUT CONDITIONS

7.1 Introduction

The steady-state chemostat culture experiments carried out in chapter five were used to determine the proportion of plasmid-free cells within a culture. The chemostat was used as a basic model of a clinical infection with the aim of showing that plasmid destabilisation could be used to reduce antibiotic resistance. However, while useful in indicating the level of instability required to remove a culture from the chemostat, it did not allow for precise measurements of the effect. Determination of growth rates allows the effects of plasmid instability to be measured in a quantitative manner.

The growth rates (μ) of cultures in steady-state are limited by the dilution rate (D) of the chemostat, such that $D = \mu$, the situation seen within cultures studied in chapter five. However, there is a maximum limit of μ, the maximum specific growth rate (μ_{max}), where organisms are dividing at their fastest rate. The measurement of μ_{max} is calculated as a value per hour, in other words, the number of divisions a single cell will undergo within a sixty-minute period. Comparing the μ_{max} of a plasmid-free culture with the μ_{max} of a plasmid-bearing culture will indicate the effect plasmid burden has on cell division, by giving the difference in the division times of the two cultures.

μ_{max} is determined by washout cultures, where the dilution rate of the chemostat is increased above the maximum specific growth rate of the cells. In this way, the culture cannot divide quickly enough in order to sustain biomass within the culture vessel, and the biomass will decrease over time. The rate of biomass loss is indicative of the μ_{max} of the culture (Pirt, 1975).

The determination of μ_{max} of an organism by washout has been used for several studies, most frequently as a tool for improving large-scale fermentations in an industrial setting. Pirt and Callow (1960) used washout as a way of determining the μ_{max} of *Penicillium chrysogenum* in order to assist in the large-scale production of penicillin. More recently, determination of μ_{max} has been used to improve the production of

Quorn® myco-protein by selecting for mutants with growth rate advantages (Simpson *et al*., 1998).

The aim of this section of work was to quantify more accurately the effect of plasmid destabilisation on the growth rate of a cell population. The assumption so far has been that an increase in plasmid-free cells within an antibiotic-containing environment would cause a decrease in the growth rate of the population, as cells would be killed or incapacitated by the action of antibiotics.

The μ_{max} of *E. coli* C2110 bearing plasmids pALA1029, pOG04, pOG4.003, pKO1029, pKO04 and pKO4.003 in both the presence and absence of antibiotics was determined. The μ_{max} of *E. coli* C2110 in the presence and absence of antibiotics was also determined.

7.2 Method

The washout experiments were carried out as described in section 2.13.4. Each washout was carried out at least twice, using a fresh culture of cells, or a fresh transformant each time. Prior to each washout, D_{crit} was determined by preliminary experiments (section 2.13.4.1). The method used for the individual washout experiments varied depending on whether a culture of plasmid-bearing cells was being studied and whether an antibiotic was added to the culture medium. Full details of each type of method are described in sections 2.13.4.2 to 2.13.4.5.

The μ_{max} of *E. coli* C2110 was determined in the absence of antibiotics and in the presence of ampicillin and chloramphenicol. The μ_{max} of *E. coli* C2110 containing plasmid pALA1029, pOG04 or pOG4.003 was determined in the presence and absence of ampicillin. The μ_{max} of *E. coli* C2110 containing plasmid pKO1029, pKO04 or pKO4.003 was determined in the presence and absence of chloramphenicol.

The μ_{max} of *E. coli* C2110 containing plasmid pOG4 or pKO4 was not determined in order to speed the data acquisition phase.

Both OD_{600} and viable count data were obtained from each washout experiment. The natural log (ln) of the data was plotted against the time in hours of the experiment. μ_{max} was calculated by adding the gradient of the line to the dilution rate used to induce washout (D + gradient = μ_{max}). Figs. 7.1, 7.2 and 7.3 include values for the gradient of the line (gradient), the dilution used to obtain washout (D), the calculated maximum specific growth rate (μ_{max}), and the correlation coefficient (R^2), which shows the accuracy of the points on the gradient line.

Once the data had been obtained for the determination of μ_{max}, the colonies present on the viable count plates were replica plated onto LB agar containing antibiotics (section 2.3.2) and antibiotic-free LB agar (section 2.3.1). This allowed determination of the percentage of plasmid-free cells within the chemostat vessel during washout (Table 7.4 and 7.7).

Mean values and 95% confidence limits were calculated (section 2.14.1) for use in Tables 7.1, 7.2, 7.3, 7.5, 7.6 and in Figs. 7.4 and 7.5. In order to determine the statistical significance between the results obtained, the Student *t*-test was used (section 2.14.2).

7.3 Results

7.3.1 Preliminary experiments

The preliminary experiments were carried out in order to simplify the complete washout experiments, and therefore full results will not be included herein. However, it is worth noting that the calculated D_{crit} for all washout experiments was approximately 1.4 h^{-1}.

7.3.2 Calculation of the μ_{max} of *E. coli* C2110 grown in the absence of antibiotics

Washout experiments to determine the μ_{max} of *E. coli* C2110 in the absence of antibiotics were carried out as described in section 2.13.5.2. Fig. 7.1 shows the viable count data obtained during a single washout experiment of *E. coli* C2110 in the absence of antibiotics. Fig. 7.2 shows the OD_{600} data obtained during the same experiment.

Both figures include data for the gradient of the slope, the dilution rate (D), the calculated μ_{max} and the correlation coefficient (R^2).

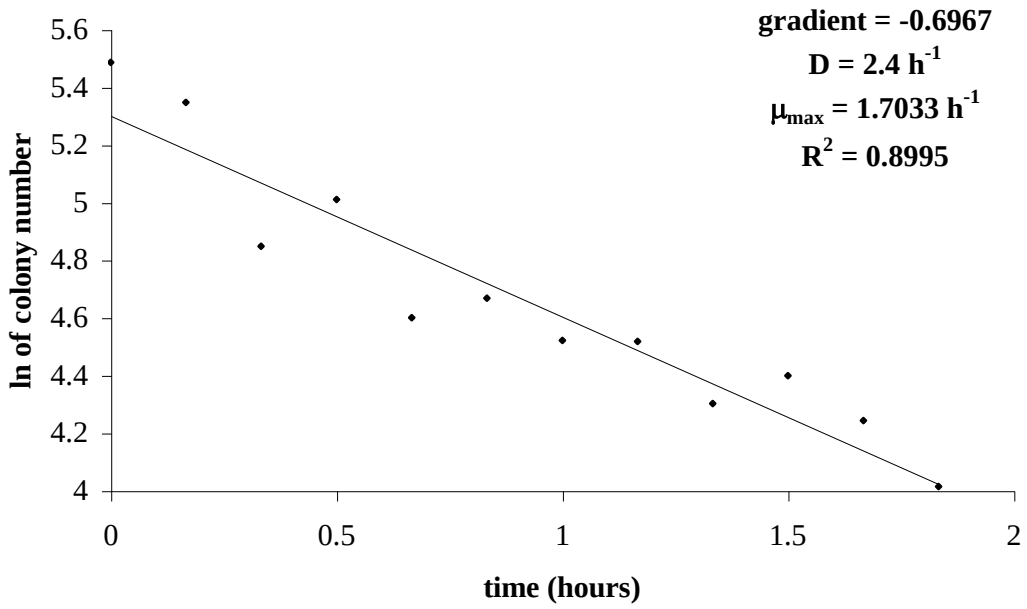


Fig. 7.1: The viable count data obtained from a washout experiment on *E. coli* C2110 grown in the absence of antibiotics

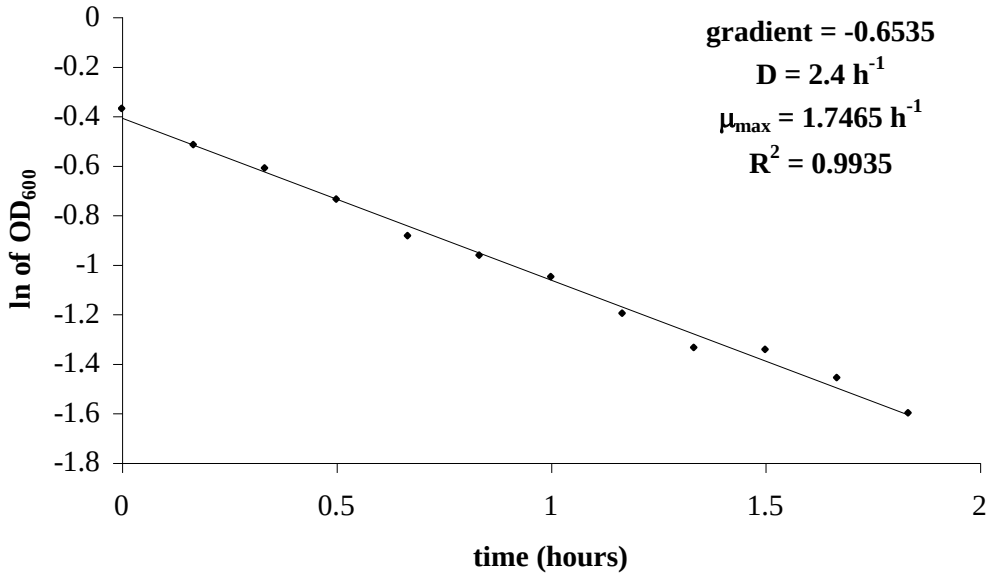


Fig. 7.2: The OD_{600} data obtained from a washout experiment on *E. coli* C2110 grown in the absence of antibiotics

Determination of the μ_{max} of *E. coli* C2110 was carried out on six separate occasions, using a fresh culture each time. For each experiment, both OD_{600} and viable count data was obtained. As the OD_{600} data showed a higher correlation coefficient, this data was used to determine the mean μ_{max} of *E. coli* C2110 in the absence of antibiotics. The individual μ_{max} results, and the mean μ_{max} of *E. coli* C2110 in the absence of antibiotics can be found in Table 7.1.

Individual results for the μ_{max} of *E. coli* C2110 calculated using OD_{600} data	**Mean μ_{max} of *E. coli* C2110 with 95% confidence limits**
1.6561 1.7202 1.7465 1.6880 1.8012 1.7066	1.7198 ± 0.053

Table 7.1: Individual results for μ_{max}, and the mean μ_{max} of *E. coli* C2110 calculated from OD_{600} data, showing 95% confidence limits

Table 7.1 shows that the μ_{max} of *E. coli* C2110 can vary between individual experiments, although the 95% confidence limits are small.

7.3.3 Calculation of the μ_{max} of *E. coli* C2110 grown in the presence of ampicillin

Washout experiments were carried out as described in section 2.13.4.3. Fig. 7.3 shows the OD_{600} data obtained during the washout of a culture of *E. coli* C2110 in the presence of ampicillin. This figure includes data for the gradient of the slope, the dilution rate (D), the calculated μ_{max} and the correlation coefficient (R^2). It can be seen that cell loss was very rapid in the presence of ampicillin, and within 30 minutes the chemostat vessel was almost completely cleared of biomass. As a result, washout experiments under these conditions are not involved with determining the maximum specific growth rate, but instead the maximum specific death rate or $ß_{max}$ (Mansour *et al*., 1993; Taormina & Beuchat, 2002).

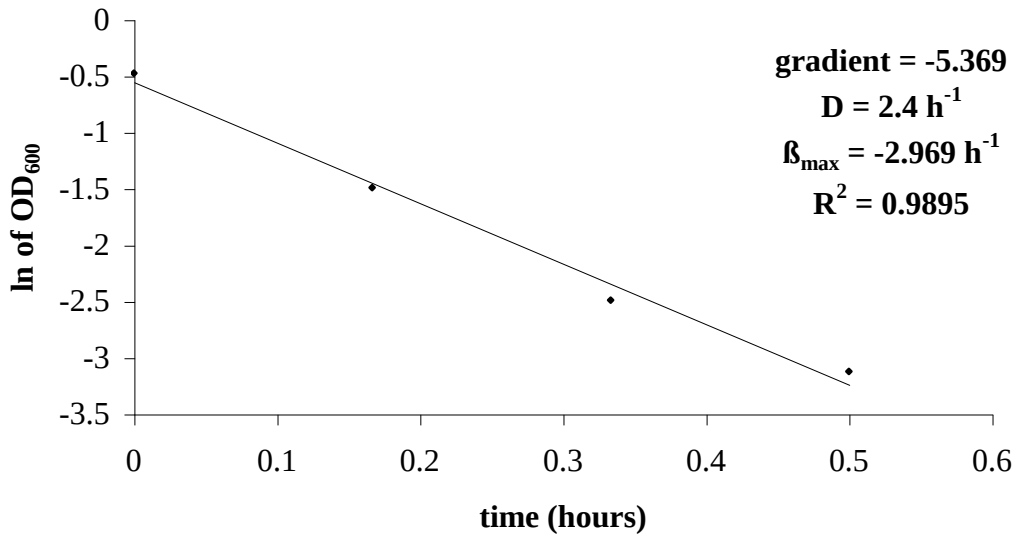


Fig. 7.3: The OD_{600} data obtained from a washout experiment on *E. coli* C2110 grown in the presence of ampicillin

Determination of the $ß_{max}$ of *E. coli* C2110 in the presence of ampicillin was carried out on ten separate occasions using a fresh culture each time. For each experiment, both OD_{600} and viable count data was obtained.

At first, washout was initiated by an increase in dilution rate at the same time as the addition of ampicillin to the culture vessel and media reservoir (2.13.4.3). Due to the rapid cell loss from the chemostat under these conditions, several experiments were run where the dilution rate was not increased, as the addition of ampicillin alone induced washout. The dilution rate for these experiments was therefore left at the value calculated during the preliminary experiments (section 2.13.4.1), which averaged at approximately 1.4 h^{-1}.

Table 7.2 includes the individual maximum specific death rates of *E. coli* C2110 in the presence of ampicillin calculated from three types of measurement. Also included are the mean $ß_{max}$ results for each of the three groups.

Measurement type and dilution rate	Individual results for the $ß_{max}$ of *E. coli* C2110 in the presence of ampicillin	Mean $ß_{max}$ of *E. coli* C2110 in the presence of ampicillin with 95% confidence limits
OD_{600} data where $D = 1.4\ h^{-1}$	-1.2260 -0.4874 -0.5649	-0.759 ± 1.008
OD_{600} data where $D = 2.4\ h^{-1}$	-1.5034 -1.7154 -2.2432 -2.9690	-2.110 ± 0.906
Viable Count data where $D = 2.4\ h^{-1}$	-7.7250 -8.3240 -7.7260	-7.925 ± 0.858

Table 7.2: Individual $ß_{max}$ results, and the mean $ß_{max}$ of *E. coli* C2110 grown in the presence of ampicillin under different dilution rates and using different styles of measurement, showing 95% confidence limits

Table 7.2 shows that the value of $ß_{max}$ can vary widely depending on whether OD_{600} or viable count data was used for the calculation. It can also be seen that the dilution rate affects the calculated $ß_{max}$. In addition there is variation between individual $ß_{max}$ results obtained under the same conditions of measurement.

There is a highly significant difference ($P < 0.1\%$) between the mean $ß_{max}$ calculated by viable count and the mean $ß_{max}$ calculated by OD_{600}, when $D = 2.4\ h^{-1}$. There is also a significant difference ($5\% > P > 2\%$) between the mean $ß_{max}$ calculated by OD_{600} when $D = 2.4\ h^{-1}$ and the mean $ß_{max}$ calculated by OD_{600}, when $D = 1.4\ h^{-1}$.

7.3.4 Calculation of the μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of ampicillin

Washout experiments were carried out as described in sections 2.13.4.4 and 2.13.4.5. Determination of the μ_{max} of *E. coli* C2110 containing the plasmid pALA1029, pOG04 or pOG4.003 grown in both the presence and absence of ampicillin was carried out on at least two occasions, using a fresh transformant each time. For each experiment, data was obtained for both viable count and OD_{600}, and as OD_{600} data showed a higher correlation coefficient, this data was used to calculate μ_{max}. Table 7.3 shows the

individual μ_{max} results, and the mean μ_{max} with 95% confidence limits for cultures of *E. coli* C2110 containing each plasmid in the presence and absence of ampicillin.

The viable count plates were replica plated in order to obtain the percentage of plasmid-free cells within the cultures during washout conditions. The results are shown in Table 7.4. This table shows that two levels of percentage sensitivity to ampicillin were obtained from experiments involving cultures of *E. coli* C2110 containing plasmid pOG04. One set of experiments showed a high percentage sensitivity to ampicillin (approximately 40% to 60%), while the other set showed a low percentage sensitivity to ampicillin (approximately 2% to 8%). This would suggest that some alteration to the plasmid had occurred between experiments, and therefore the experiments that showed a high percentage of resistance were classified as containing type one pOG04, while those showing a low level of resistance were classified as containing type two pOG04.

As a result of the differences in resistance to ampicillin following replica plating, the μ_{max} values from the experiments involving plasmid pOG04 were also divided into two groups, the first containing experiments carried out with type one pOG04, the second containing experiments carried out with type two pOG04. The results are shown in Table 7.3 as separate groups as a result of this, although the actual values of μ_{max} for these two experimental groups are very similar.

Plasmid	Presence / Absence of Ampicillin	Individual results	Mean μ_{max} calculation with 95% confidence limits
pALA1029	Absent	1.7707 1.7571	1.764 ± 0.086
pALA1029	Present	1.7440 1.6680	1.706 ± 0.483
pOG4.003	Absent	2.0266 1.9619 1.8259 1.9849	1.950 ± 0.138
pOG4.003	Present	1.3878 1.4434 1.4847	1.439 ± 0.121
Type one pOG04	Absent	1.8239 1.7668 1.7712	1.787 ± 0.079
Type one pOG04	Present	1.6189	1.6189
Type two pOG04	Absent	1.8252	1.8252
Type two pOG04	Present	1.6620	1.6620

Table 7.3: Individual results for μ_{max}, and the mean μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of ampicillin calculated from OD_{600} data, showing 95% confidence limits

Plasmid	Presence / Absence of Ampicillin	Average percentage sensitivity of the cultures to ampicillin with 95% confidence limits
pALA1029	Absent	0.75 ± 0.89
pALA1029	Present	0.60 ± 1.23
Type one pOG04	Absent	61.2 ± 5.25
Type one pOG04	Present	43.2 ± 8.02
Type two pOG04	Absent	7.27 ± 3.79
Type two pOG04	Present	2.34 ± 1.77
pOG4.003	Absent	46.7 ± 5.18
pOG4.003	Present	26.7 ± 3.07

Table 7.4: A table to show the overall percentage sensitivity to ampicillin of cultures of *E. coli* C2110 containing plasmids grown in the presence or absence of ampicillin

Table 7.4 shows that all the plasmid-bearing cultures show a higher percentage sensitivity to antibiotics when grown in the absence of ampicillin than they do when grown in the presence of ampicillin.

The mean μ_{max} calculated for each plasmid-containing culture in the presence and absence of ampicillin has been used to produce the bar chart shown in Fig. 7.4.

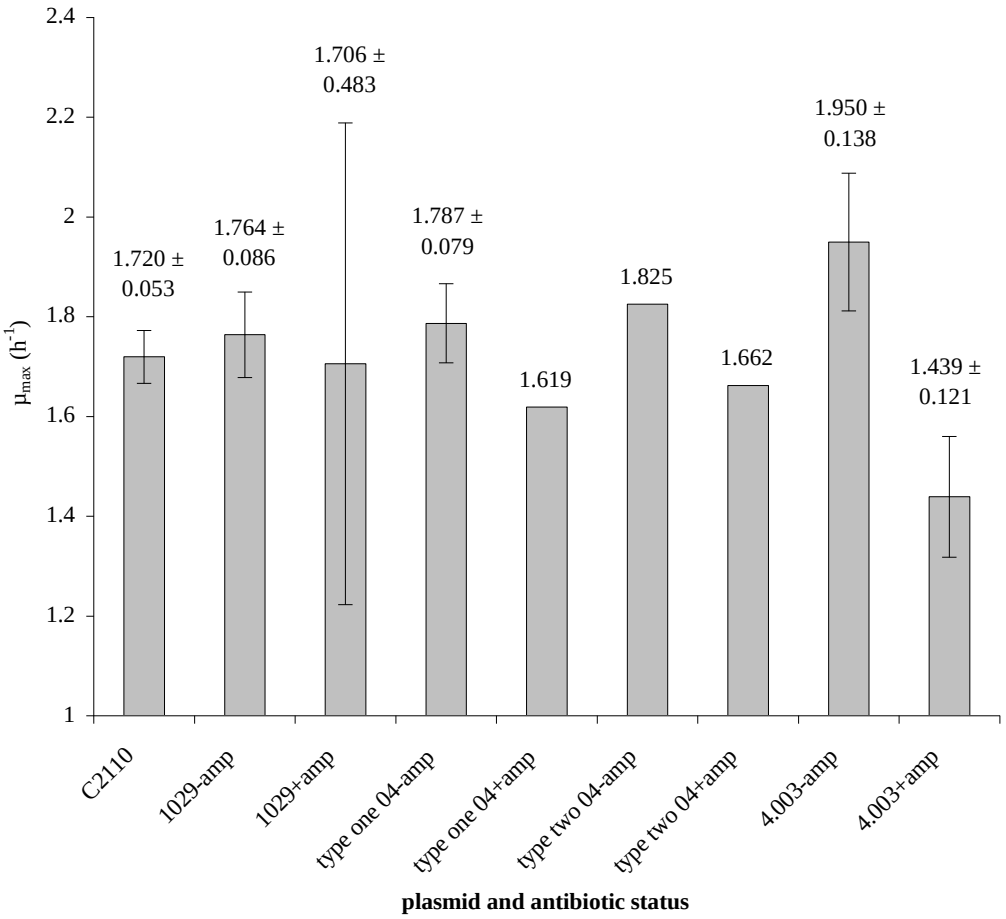


Fig. 7.4: A bar chart to show the mean μ_{max} of *E. coli* C2110 grown in the absence of ampicillin, and the mean μ_{max} of *E. coli* C2110 containing plasmids grown in the presence and absence of ampicillin, showing 95% confidence limits

It can be seen in Fig. 7.4 that many of the error bars obtained from the 95% confidence limits overlap. There are, however, significant differences between some of the results.

The μ_{max} of *E. coli* C2110 is significantly (1% > P > 5%) lower than the μ_{max} of *E. coli* C2110 containing plasmid pOG04 in the absence of ampicillin. The μ_{max} of *E. coli* C2110 is highly significantly (P < 0.1%) lower than the μ_{max} of *E. coli* C2110 containing plasmid pOG4.003 in the absence of ampicillin.

The μ_{max} of *E. coli* C2110 is highly significantly (P < 0.1%) higher than the μ_{max} of *E. coli* C2110 containing plasmid pOG4.003 in the presence of ampicillin.

The μ_{max} of *E. coli* C2110 containing plasmid pOG4.003 in the absence of ampicillin is highly significantly (P < 0.1%) higher than the μ_{max} of *E. coli* C2110 containing plasmid pOG4.003 in the presence of ampicillin.

Although not statistically significant, a pattern can be observed within the mean μ_{max} values. In the absence of ampicillin, cultures containing the less stable plasmids, such as pOG4.003, have a higher mean μ_{max} than that of cultures containing the more stable plasmids. In the presence of ampicillin, cultures containing the less stable plasmids have a slower growth rate than that of cultures containing the more stable plasmids. In the absence of ampicillin, a culture of *E. coli* C2110 that does not contain a plasmid shows a slower growth rate than all of the plasmid-bearing cultures.

7.3.5 Calculation of the μ_{max} of *E. coli* C2110 grown in the presence of chloramphenicol

Washout experiments were carried out as described in section 2.13.4.3. For each experiment OD_{600} and viable count data were collected. The washout of *E. coli* C2110 in the presence of chloramphenicol was rapid, although not as rapid as the washout seen in experiments involving *E. coli* C2110 in the presence of ampicillin (section 7.3.3). Several experiments were run where the dilution rate was not increased, as the addition of chloramphenicol alone induced washout. The dilution rate for these experiments was therefore left at the value calculated during the preliminary experiments (section 2.13.4.1) which averaged at approximately 1.4 h^{-1}.

Table 7.5 includes the individual μ_{max} results of *E. coli* C2110 in the presence of chloramphenicol calculated from OD_{600} data taken at a dilution rate of 2.4 h^{-1} and at

1.4 h^{-1}. The individual $ß_{max}$ results of *E. coli* C2110 in the presence of chloramphenicol calculated from viable count data taken at a dilution rate of 2.4 h^{-1} are also included. The mean μ_{max} and $ß_{max}$ results for each of the three groups of measurements are also given in Table 7.5.

Measurement type and dilution rate	**Individual μ_{max} / $ß_{max}$ results for *E. coli* C2110 in presence of chloramphenicol**	**Mean μ_{max} / $ß_{max}$ for *E. coli* C2110 in presence of chloramphenicol with 95% confidence limits**
OD_{600} data where D = 2.4 h^{-1}	0.3348 0.3044	0.3196 ± 0.193
OD_{600} data where D = 1.4 h^{-1}	0.2881 0.5565 0.4538 0.4144 0.2473 0.4006	0.3930 ± 0.118
Viable Count data where D = 2.4 h^{-1}	-1.9080 -1.1508	-1.5290 ± 4.810

Table 7.5: Individual μ_{max} / $ß_{max}$ results, and the mean μ_{max} / $ß_{max}$ of *E. coli* C2110 grown in the presence of chloramphenicol under different dilution rates and using different styles of measurement, showing 95% confidence limits

There is no significant difference between the μ_{max} of *E. coli* C2110 when calculated from measurements taken at D = 2.4 h^{-1} or D = 1.4 h^{-1}. There is also no significant difference between the μ_{max} obtained from viable count data and that obtained from OD_{600} data, although the mean μ_{max} calculated from viable count data is considerably lower than the mean μ_{max} calculated from OD_{600} data. There is, however, considerable variation between the individual μ_{max} and $ß_{max}$ results.

7.3.6 Calculation of the μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of chloramphenicol

Washout experiments were carried out as described in sections 2.13.4.4 and 2.13.4.5. Determination of the μ_{max} of *E. coli* C2110 containing each of the plasmids pKO1029, pKO04 and pKO4.003 in both the presence and absence of chloramphenicol was carried

out on at least two occasions, using a fresh transformant each time. For each experiment, data was obtained for both viable count and OD_{600}, and as OD_{600} data showed a higher correlation coefficient, this data was used to calculate μ_{max}. Table 7.6 shows the individual μ_{max} results, and the mean μ_{max} with 95% confidence limits for cultures of *E. coli* C2110 containing each plasmid in the presence and absence of chloramphenicol.

Plasmid	**Presence / Absence of Chloramphenicol**	**Individual results**	**Mean μ_{max} calculation with 95% confidence limits**
pKO1029	Absent	1.7178 1.7098	1.714 ± 0.0508
pKO1029	Present	1.7126 1.6939	1.703 ± 0.119
pKO4.003	Absent	1.8909 1.9688 1.7473 1.8120	1.855 ± 0.153
pKO4.003	Present	1.4709 1.7235 1.4793 1.3942	1.517 ± 0.227
pKO04	Absent	1.7879 1.8061	1.797 ± 0.116
pKO04	Present	1.2401 1.3600 1.1814 1.0307	1.203 ± 0.218

Table 7.6: Individual results for μ_{max}, and the mean μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of chloramphenicol calculated from OD_{600} data, showing 95% confidence limits

The viable count plates were replica plated onto LB agar containing chloramphenicol (section 2.3.2) and LB agar (section 2.3.1) in order to obtain the percentage of plasmid-free cells within the cultures during washout conditions. The results for percentage sensitivity to chloramphenicol of the cultures are shown in Table 7.7.

Plasmid	Presence / Absence of Chloramphenicol	Average percentage sensitivity of the cultures to chloramphenicol with 95% confidence limits
pKO1029	Absent	0.60 ± 0.43
pKO1029	Present	0.45 ± 0.74
pKO04	Absent	64.4 ± 6.63
pKO04	Present	40.0 ± 6.14
pKO4.003	Absent	54.6 ± 4.23
pKO4.003	Present	22.8 ± 2.98

Table 7.7: A table to show the overall percentage sensitivity to chloramphenicol of cultures of *E. coli* C2110 containing plasmids grown in the presence or absence of chloramphenicol

Table 7.7 shows that the percentage sensitivities obtained from the cultures containing plasmid pKO04 were high (approximately 40% to 60%), similar to the results obtained from experiments involving the type one pOG04 plasmid (Table 7.4). No experiments involving plasmid pKO04 were seen to show the low percentage sensitivity to antibiotics of experiments involving type two pOG04.

Table 7.7 shows that in the absence of chloramphenicol, all of the plasmid-bearing cultures show higher percentage sensitivity to antibiotics than they do in the presence of chloramphenicol.

The mean μ_{max} calculated for each plasmid-containing culture in the presence and absence of chloramphenicol has been used to produce the bar chart shown in Fig. 7.5.

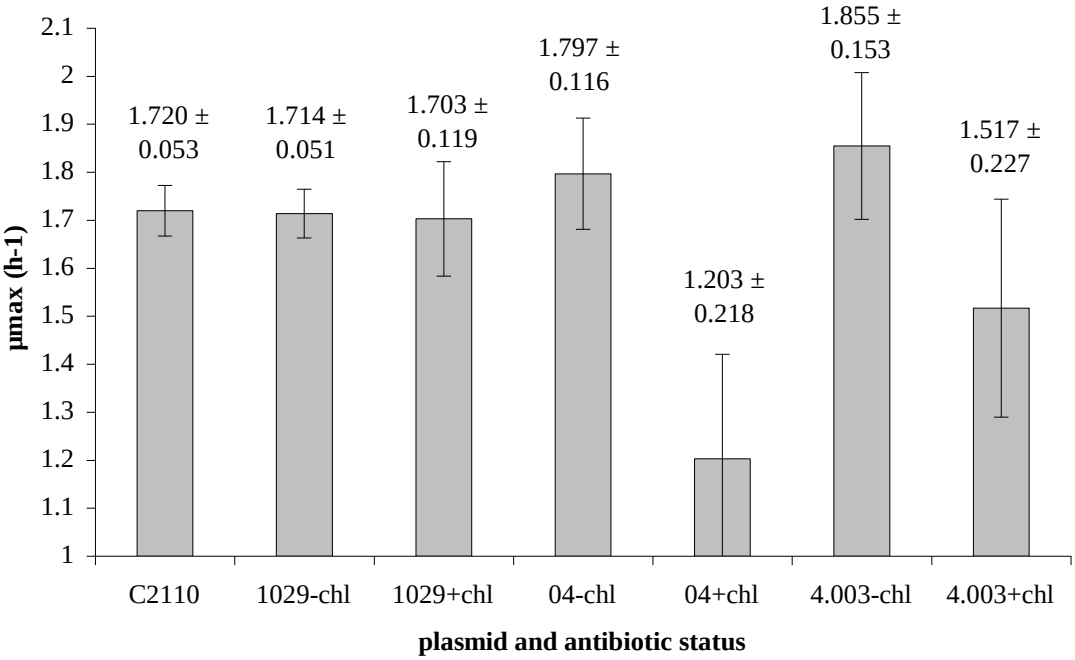


Fig. 7.5: **A bar chart to show the mean μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of chloramphenicol, showing 95% confidence limits**

It can be seen in Fig. 7.5 that many of the error bars obtained from the 95% confidence limits overlap. There are, however, significant differences between some of the results.

The μ_{max} of *E. coli* C2110 containing plasmid pKO04 in the presence of chloramphenicol is highly significantly ($P < 0.1\%$) lower than the μ_{max} obtained from any other experiment. In addition, the μ_{max} of *E. coli* C2110 containing plasmid pKO4.003 in the presence of chloramphenicol is significantly ($1\% > P > 5\%$) lower than the μ_{max} of *E. coli* C2110 containing plasmids pKO4.003 in the absence of chloramphenicol.

7.4 Discussion

7.4.1 The μ_{max} of *E. coli* C2110

Fig. 7.1 displays the μ_{max} of *E. coli* C2110 determined from viable count data as 1.7033 h^{-1}, while Fig. 7.2 displays the μ_{max} of *E. coli* determined from OD_{600} data as 1.7465 h^{-1}. Both types of data were obtained from the same experiment, and therefore the true μ_{max} of the culture cannot be different. As a result, the difference in the calculated result must be because of difference in the type of data obtained. Viable count data will only record the presence of living cells, while OD_{600} readings will record the density of the whole culture, which will include any dead cells, or cellular matter.

It may be considered that viable count readings would be more appropriate for the purpose of this study, as living cells are more appropriate for the determination of the growth rate. However, the variability of the viable count data is so great that it is likely that more inaccuracies in the μ_{max} calculation would be seen as a result.

Therefore, as a result of using the more accurate OD_{600} data, the variability of individual μ_{max} results shown in Table 7.1 is surprising. The OD_{600} data collected from each individual experiment showed a remarkable correlation coefficient; all individual data points showed at least 95% accuracy to the calculated gradient, suggesting the data collected for each experiment was precise. However, when the individual results were compared to each other, there was a large amount of variation. The explanation for this is that differences occur within individual washout experiments, and therefore the calculation of μ_{max} is not repeatable. This phenomenon is discussed in more detail in section 7.4.6.

Another point of interest is the difference between the result obtained for D_{crit} during the preliminary experiments (section 7.3.1) and the final μ_{max} value (Table 7.1). The actual D_{crit} determined for *E. coli* C2110 was 1.47 h^{-1}, while the mean μ_{max} of *E. coli* C2110 was 1.7198 ± 0.053 h^{-1}. There is a large difference between the expected value of μ_{max} as suggested by the preliminary studies and the value obtained from washout experiments. This was observed in all the experiments described throughout this chapter.

7.4.2 The $ß_{max}$ of *E. coli* C2110 grown in the presence of ampicillin

It was expected that addition of ampicillin to a culture of sensitive cells would cause a remarkably fast washout of the culture from the chemostat. In order to reduce the variability between experiments, the first washouts of this type were carried out in the same way as all other experiments, in that the dilution rate was increased at the start of washout. However, the loss of cells was so rapid that the decision was made to leave the dilution rate unaltered during the washout phase so that the culture could be observed over a longer period of time. This clearly makes a large difference to the value of $ß_{max}$ as seen in Table 7.2.

It is not surprising that the calculated values of $ß_{max}$ vary between viable count data and OD_{600} data. As stated in section 7.4.1, OD readings will include the density of dead cell matter, and there would be a large amount of this within a sensitive culture subjected to a bactericidal antibiotic like ampicillin. However, the difference between the $ß_{max}$ calculated using OD_{600} data at the different dilution rates was not expected. The $ß_{max}$ for a specific organism under identical conditions will always be the same, and the culture medium, pH, temperature and concentration of ampicillin were the same for both experiments.

The main difference between the experiments was the dilution rate. This difference should be negated by the calculation for $ß_{max}$ (D + gradient = $ß_{max}$), although this is clearly not the case. A possible reason for this difference is discussed in detail in section 7.4.6.

7.4.3 The μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of ampicillin

The main problem with discussing the data obtained in section 7.3.4 is the lack of statistical significance between the results, due to the variability of washout experiments.

A significant difference between the μ_{max} of a culture containing pALA1029 grown in the presence of ampicillin and the μ_{max} of a culture containing pOG4.003 grown in the presence of ampicillin would show that plasmid destabilisation can affect the growth

rates of cultures under antibiotic pressure. However, the error bars obtained from experiments involving pALA1029 in the presence of ampicillin are large, and the mean μ_{max} of *E. coli* C2110 containing pALA1029 in the presence of ampicillin is not significantly different to any of the other results. It can be observed that the mean μ_{max} of *E. coli* C2110 containing pALA1029 in the presence of ampicillin is higher than any of the other plasmid-bearing cultures in the presence of ampicillin, but this has been shown not to be statistically significant.

The μ_{max} of *E. coli* C2110 in the absence of ampicillin is significantly higher than the μ_{max} of *E. coli* C2110 containing pOG4.003 in the presence of ampicillin. This may suggest that the growth rate of the culture containing pOG4.003 has been affected by plasmid destabilisation, however, there are many variables between the two experiments including the presence of plasmid burden and antibiotic pressure in the culture containing pOG4.003. As a result, no direct comparison can be made between the two sets of results.

The μ_{max} of *E. coli* C2110 containing pOG4.003 in the absence of ampicillin is significantly higher than the μ_{max} of *E. coli* C2110 containing pOG4.003 in the presence of ampicillin. This shows that there is a reduced growth rate of this culture in the presence of ampicillin, probably because of the production of plasmid-free cells as a result of plasmid destabilisation.

A comparison between the μ_{max} of the plasmid-bearing cultures in the absence of ampicillin and the μ_{max} of *E. coli* C2110 in the absence of ampicillin highlights an interesting result (Fig. 7.4). The μ_{max} of *E. coli* C2110 is significantly lower than the μ_{max} of *E. coli* C2110 containing pOG04 or pOG4.003 in the absence of ampicillin. A number of studies have shown that plasmid-containing cultures have lower growth rates than plasmid-free cultures, suggesting that plasmid-bearing cultures are at a competitive disadvantage in the absence of selection pressure (Godwin & Slater, 1979; Kyslik *et al.*, 1993). In the same way it has been assumed throughout this study that the plasmids place a metabolic burden on the host cell, and that the benefits of retaining these plasmids are only seen under antibiotic pressure. The results obtained from this set of washout experiments indicate that the plasmids are potentially providing the host cell

with growth benefits in the absence of antibiotics. The data collected during this study cannot be used to explain this situation.

This phenomenon has been observed previously, where a culture of *E. coli* B containing the plasmid pACYC184 had a competitive advantage over a plasmid-free culture, due to alterations within the host strain (Lenski *et al*., 1994). However, this increased fitness was only observed after 500 generations of growth, and as these experiments only observed the culture over approximately 3 generations of growth, it is unlikely that the host strain has mutated in this short space of time. Another study involving *E. coli* strain K12 containing plasmid TP120 showed that during chemostat growth at least one of the resistance markers on this plasmid was lost during each experiment. It was suggested that the plasmid fragmented and lost portions of the DNA, reducing the plasmid burden on the host, allowing the host a selective advantage in a mixed culture (Godwin & Slater, 1979).

Although not statistically significant, the mean μ_{max} of *E. coli* C2110 containing plasmid pOG4.003 in the absence of ampicillin is higher than the mean μ_{max} of *E. coli* C2110 containing plasmid pALA1029. This is almost certainly due to a reduction in plasmid burden, which is confirmed by the percentage of ampicillin sensitive cells within the populations (Table 7.4). While over 98% of the cells within the culture containing pALA1029 are plasmid-bearing, approximately 50% of the cells within the culture containing pOG4.003 are plasmid-free. However, the suggestion of a reduction in plasmid burden at this point contradicts the above theory that the plasmids studied in this work may confer a benefit on the host cell in the absence of antibiotics.

The data obtained from replica plating of the viable count plates yielded some other interesting results (Table 7.4). First, there is clear evidence that the presence of ampicillin is destroying plasmid-free cells. In all experiments, the percentage of plasmid-free cells within a culture in the absence of ampicillin was higher than the percentage of plasmid-free cells in the presence of ampicillin. In some cases, for example in cultures containing plasmid pOG4.003, the percentage of sensitive cells is almost halved when the culture is grown in the presence of ampicillin.

An important observation from the replica plating results was that, at some point, there has been an alteration in the stability of plasmid pOG04. The washout experiments showed that all pOG04-bearing cultures grown in the presence of ampicillin had a similar μ_{max} (Table 7.3), however, one type of culture showed almost 50% sensitivity to ampicillin, while the other type showed only 3% sensitivity to ampicillin. It was expected that under fast-growth-rate conditions, the stability of plasmid pOG04 would not show the high sensitivity seen in steady-state chemostat culture experiments (section 5.4.5), and would return to the levels seen in batch culture experiments (section 4.4.2). The μ_{max} results suggest this is the case, with the μ_{max} of cultures containing pOG04 falling between those determined for cultures containing pALA1029 and pOG4.003. However, the percentage resistances suggest otherwise. The type one pOG04 cultures show a higher level of sensitivity to ampicillin than that seen in the cultures containing pOG4.003, a similar effect to that seen under steady-state chemostat conditions. It therefore cannot be ruled out that structural changes have taken place within this plasmid over the course of this study, although this cannot be confirmed without further work, as no changes are evident in the post-experimental restriction digests (section 2.13).

The type one pOG04 plasmid showing the high sensitivity to ampicillin is likely to be the altered plasmid, as the percentage sensitivity to ampicillin of the type two cultures falls between the percentage sensitivities of the cultures containing plasmids pOG1029 and pOG4.003, a similar result to that obtained during batch culture experiments (chapter four). However, steady-state chemostat culture experiments (chapter five) have been shown to lead to the selection of plasmids with a naturally increased stability (Seegers *et al*., 1995). Therefore, it is possible that the type one plasmid is the unaltered plasmid, and the stability level seen in batch culture experiments is unusual as a result of growth rate differences, or medium alterations. This would mean that the stable type two plasmid has occurred as a result of natural selection for a more stable plasmid.

7.4.4 The μ_{max} / $ß_{max}$ of *E. coli* C2110 grown in the presence of chloramphenicol

Chloramphenicol is described as a bacteriostatic antibiotic; it does not kill cells, but merely prevents replication. However, the results obtained in section 7.3.5 would suggest otherwise. During the experiments to determine the $ß_{max}$ of *E. coli* C2110 in the

presence of ampicillin two different dilution rates were used, and this was repeated in order to obtain the μ_{max} / $ß_{max}$ of *E. coli* C2110 in the presence of chloramphenicol.

The first important point to make is that both values of μ_{max} obtained from calculations using OD_{600} data are positive, suggesting that the cells are able to grow even with the presence of an inhibitory concentration of chloramphenicol. As this clearly cannot be the case, this corroborates the theory that OD_{600} data includes the presence of dead cells and cellular matter, not just the presence of living cells. Secondly, the results obtained from viable count data lead to a negative 'growth rate', in other words a $ß_{max}$ value. This suggests that chloramphenicol can kill cells over an extended period of time. This is not altogether surprising, for although cells can survive without actively replicating, some amount of transcription and translation is necessary for general 'housekeeping' functions, otherwise known as maintenance energy (Pirt, 1965; Pirt, 1982; Wallace & Holms, 1986). Although cells could survive for a limited period of time without any protein production, the results obtained here suggest that the exposure of cells to chloramphenicol over the two-hour duration of the experiments will cause cell death.

7.4.5 The μ_{max} of *E. coli* C2110 containing plasmids grown in the presence or absence of chloramphenicol

Many of the comments made in section 7.4.3 regarding the plasmid-containing cultures are relevant here and therefore will not be reiterated in depth. Again, a serious limitation regarding the discussion of these results is a lack of statistical significance between the data obtained.

One significant result is that the μ_{max} of cultures containing pKO4.003 in the absence of chloramphenicol is higher than the μ_{max} of this culture in the presence of chloramphenicol (Fig. 7.7). This is almost certainly due to the large number of plasmid-free cells within the culture, a supposition supported by the results obtained for percentage sensitivity to chloramphenicol during replica plating tests (Table 7.7).

Another significant result is that the μ_{max} of *E. coli* C2110 containing pKO04 in the presence of chloramphenicol is significantly lower than the μ_{max} obtained for any other experiment. Only one level of sensitivity to chloramphenicol was observed for these

cultures, unlike the experiments involving *E. coli* C2110 containing plasmid pOG04. The percentage sensitivity to chloramphenicol of these cultures was 65%, a higher percentage sensitivity than that seen for *E. coli* C2110 containing pKO4.003. Also, in contrast to the mean μ_{max} of *E. coli* C2110 containing pOG04 in the presence of ampicillin, this high level of sensitivity appears to be affecting the growth rate of the culture (Fig. 7.5). No explanations can be given for these results with the current data.

Structural tests on both pOG04 and pKO04 may show that a mutation has occurred, although the same mutation occurring on separate plasmids is highly unlikely. If the mutation was present before plasmid pKO04 was constructed, aberrant results should have been observed during the batch culture experiments. As this was not the case it seems unlikely that the mutation was present before this study was commenced.

7.4.6 The limitations of washout as a method for determining μ_{max}

No comparison has been made in this chapter of the results obtained for the ampicillin-resistance-conferring plasmids and the chloramphenicol-resistance-conferring plasmids, as there was in chapters four and five. This is purely due to the lack of statistical significance between the results obtained. The following section is an attempt to explain why the determination of μ_{max} is highly unrepeatable, which leads to large 95% confidence limits when trying to apply statistical tests.

Several papers have been published that are involved with determining μ_{max}, and none of those studied suggest replication of washout experiments in order to obtain more accurate results, or statistical methods for determining the significance between the results (Esener *et al*., 1981, Borzani, 1994, Simpson *et al*., 1998; Gupthar *et al*., 2000).

Further consideration of the definition of μ_{max} suggests the reason why this is the case. μ_{max} is defined as the maximum specific growth rate of an organism under 'a specific set of conditions'. These conditions would include the media formulation, the temperature and the pH, and while all of these are tightly controlled, it would be impossible to replicate exactly the same conditions over several experiments (Button, 1985). For example, every prepared batch of media will contain slight differences in the amount of dry powder used or the amount of the water added to dissolve the

powder; even the autoclave may alter the media components if the length of a run is a few minutes different. These differences will affect the μ_{max} of a culture, and for that reason these experiments are unrepeatable. For most experiments, increasing the number of replicates will decrease the deviation around the mean result, but for washout experiments, replicates will only serve to increase the error (Borzani, 1994; Gupthar *et al*., 2000).

Esener *et al*. (1981) published an in-depth study on washout experiments and the most efficient way of determining μ_{max}. One of the most important points raised in this publication is that traditional methods for determining μ_{max} (such as the type used in this study) rely on the culture instantly transitioning from a below-maximum growth rate in steady-state conditions to the maximum growth rate when the dilution is increased. This cannot occur, as any culture will require a period of time to adjust to a new growth rate (Tempest, 1970), and the way in which the transition is implemented will influence the calculation of μ_{max}.

Dramatically increasing the dilution rate to induce washout (as used in this study) can cause physiological shock to the cells and may cause a faster washout than the true value at the start of the experiment while the culture adjusts to the new growth rate. The other option is to increase the dilution rate by a small degree; however, the cells may be substrate limited at the start of the experiment and this will increase the length of time the culture takes to reach μ_{max} (Esener *et al*., 1981).

The solution to this problem is to balance both aspects by having a steady-state culture growing as close to D_{crit} as possible to prevent physiological shock when the dilution rate is increased, and then increasing the dilution rate sufficiently slowly to prevent substrate limitation from occurring. This is obviously a time consuming process, requiring a large amount of preliminary work to be carried out before the actual washout experiment (Esener *et al*., 1981).

An experiment carried out by Esener *et al*. (1981) demonstrated the effect of physiological shock caused by a dramatic increase in the dilution rate in order to cause washout. Their observation was that the cells washed out faster at the start of the experiment before becoming more stable at a slower washout rate, although the dilution

rate remained constant at all times. Fig. 7.6 shows an example of a washout experiment carried out during the course of this present study, where this effect can be clearly seen.

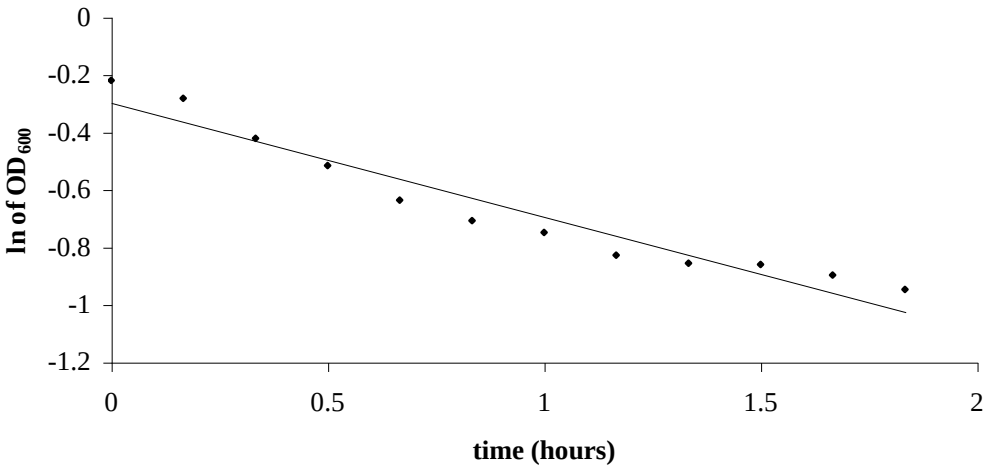


Fig. 7.6: The OD_{600} data obtained from a washout experiment on *E. coli* C2110 containing pOG4.003 grown in the absence of ampicillin

Therefore, it is possible that the method used to determine μ_{max} during this work was not the most appropriate. It appears that the cultures may have been affected by physiological shock as a result of the large increase in dilution rate, and as a result the figures obtained for μ_{max} may not be accurate. This theory is further confirmed by the difference in values obtained during the preliminary experiments, and those obtained during the washout experiments.

7.5 Conclusion

The results obtained in this section of work show that a culture containing a destabilised plasmid has a reduced growth rate in the presence of antibiotics than a culture containing a stable plasmid. This is based on observation and not statistical tests, as the method for determining μ_{max} used in this study does not lend itself to statistical analysis.

Other significant findings from this section of work include an increased knowledge of the limitations of washout experiments. While, in general, a scientific investigation requires analysis of several sets of data, determination of the μ_{max} of a culture is not a

directly repeatable experiment and therefore multiple data sets are unobtainable. Careful preliminary work, such as determining a much more specific value for D_{crit}, would reduce the variables and may therefore reduce variability between experiments allowing statistical tests.

This section of work has also shown that plasmids are inherently more unstable in the absence of antibiotic selection pressure, proved by an increase in antibiotic resistance of those cultures grown in the absence of antibiotics. It also appears that the presence of the plasmids studied in this work provide the host cell with a growth benefit, seen by an increased μ_{max} in plasmid-containing cultures in the absence of antibiotics when compared with the host cell alone.

All these observations are glimpses into a complicated system with a large number of variables, all of which could be resolved by further study and analysis. However, a conclusion can still be drawn that plasmid destabilisation does appear to affect the growth rate of plasmid-bearing cultures in the presence of antibiotics, this is encouragement enough that plasmid destabilisation is an approach that may hold future promise for new therapies against antibiotic resistant bacteria.

CHAPTER EIGHT – FURTHER WORK AND FINAL CONCLUSION

The discussions in each of the previous chapters have highlighted the fact that the work carried out during the course of this study has raised more questions than answers, and that most of these questions cannot be conclusively resolved without further research. Therefore, the purpose of this final chapter is to summarise the areas in which further research could provide insights into the unexplained results obtained during this study, and suggest ways in which this research may be carried out.

8.1 Questions that have been raised as a direct result of work carried out in this study

Many of the unusual results have been discussed in depth within the relevant chapters, along with details of individual experiments suggested for further study. In this section, the main areas of uncertainty are reiterated and a general approach for future work is described.

8.1.1 Why do the chloramphenicol-resistance conferring plasmids show a higher stability in batch culture than the ampicillin-resistance conferring plasmids?

In chapter four, section 4.4, several reasons were given for the possible increase in stability of the chloramphenicol-resistance-conferring plasmids. Most of these related to a possible change in either the values of R or $d\mu$ of the plasmids caused by the insertion of the chloramphenicol resistance gene. Determination of the actual numerical values of R or $d\mu$ of the plasmids would reduce the number of hypotheses presented, allowing for more detailed work to be carried out in the correct area. The methods for obtaining numerical values of R and $d\mu$ are described in detail in Cooper *et al*. (1987). In general, the method involves determining the increase in plasmid-free cells over the course of the entire experiment, with a linear or exponential increase being indicative of the way in which plasmid-free cells are produced.

Following this work, the experiments described in chapter four, section 4.4.5, could be carried out with a more definite purpose in mind. For example, the copy numbers of the

plasmids could be quantitatively determined (chapter three, section 3.6) proving or disproving whether there had been an increase in copy number as a result of the insertion of the DNA coding for chloramphenicol resistance.

This work might also provide the answer to the question posed in section 8.1.3 as to why some of the chloramphenicol resistance-conferring plasmids showed a higher instability than the ampicillin-resistance-conferring plasmids under steady-state chemostat culture conditions; the direct opposite to that seen in batch culture.

8.1.2 Why do plasmids pOG04 and pKO04 show such a high instability in chemostat culture?

The very high number of plasmid-free cells produced during steady-state chemostat culture of *E. coli* C2110 containing either pOG04 or pKO04 was completely unexpected. The washout experiments carried out in chapter six raised concerns that these unusual results may not be caused by a change in growth conditions, but rather that structural alterations of the plasmids had taken place.

Structural analysis of the plasmids could help to indicate whether mutations had taken place, although these mutations must have been small, or they would have been seen during the post-experimental plasmid digests (section 2.13). Changes in the plasmid copy number may also have affected the stability; quantitative determination of the copy numbers as described in chapter three, section 3.6 would resolve this suggestion.

It is possible that the cause of increased instability is the change in growth media between the batch and chemostat experiments. The batch culture experiments were carried out using full strength LB broth, but the chemostat experiments were carried out using half-strength LB broth in order to reduce biomass. Repeating the experiments using the same media for all experiments may produce different results. Minimal medium (Larkin & Day, 1985) is a low nutrient medium that dramatically reduces the growth rates of cultures. This would probably cause changes in plasmid stability levels as a result of the lack of nutrients provided. The low nutrient conditions of minimal medium could also be considered to bring the steady-state chemostat culture model

closer to the conditions found within a clinical infection, as nutrient supplies in the body are limited due to competition with other microbes and the body itself.

An important point to make is that many of the results within this study are interpreted on the basis of the assumption that the order of stabilities obtained from batch culture experiments are correct. It is possible that the stabilities shown in chemostat culture are the more accurate, and therefore the batch culture results for pOG04 and pKO04 are the more unusual results. After all, an immediate assumption could be that any stability system, even a faulty system, would be better than no stability system at all. This hypothesis could be tested by further analysis of other plasmids containing differing stability systems.

8.1.3 Why do some of the chloramphenicol-resistance conferring plasmids show a higher instability in chemostat culture than the ampicillin-resistance conferring plasmids?

Under steady-state chemostat culture conditions, the stability of pKO1029 is higher than pALA1029, reflecting the results seen in batch culture. However, the stabilities of plasmids pOG4 and pKO4 are almost identical in chemostat culture, plasmid pKO4.003 shows a higher instability than pOG4.003, and pKO04 shows a much higher instability than pOG04. This is in direct contradiction of the data obtained from batch culture experiments.

Why has there been a change in stabilities for some plasmids, but not for all? The results for plasmids pKO04 and pKO4.003 now fit the theory of higher plasmid burden of the chloramphenicol plasmids, but plasmids pKO1029 and pKO4 still do not. The exact constitution of the plasmids needs to be carefully studied to determine whether this is as a result of mutation or copy number change. In addition, further work on the concentration of antibiotics within the culture vessel may assist in interpretation of these results (chapter six). It would be expected that the more stable plasmids would be producing higher concentrations of resistance enzymes, which may be causing the unexpected results by reducing the concentration of antibiotics within the vessel to a sub-inhibitory level.

8.1.4 Why do plasmid-bearing cells show a higher μ_{max} than plasmid-free host cells in the absence of antibiotics?

It has been stated in many studies that plasmid-bearing cells always have a higher metabolic burden than plasmid-free cells. In the absence of antibiotics, these plasmids should not be providing the host cell with any benefits, as their sole function is to provide antibiotic resistance. However, the plasmids must be providing additional benefits that are as yet unknown. The discovery of the reason for this phenomenon may be difficult, if not impossible to determine. This is corroborated with the amount of published work focusing on cryptic plasmids that do benefit the host cell, but in an as yet unknown manner.

8.1.5 Is μ_{max} determination an appropriate analysis for the effect of plasmid destabilisation?

As described in chapter seven, section 7.4.6, the determination of μ_{max} is an unrepeatable experiment. While each individual experiment gives an honest result, these cannot be compared as each experiment will suffer from variability and as a result will lead to genuinely different μ_{max} calculations. The variability of the experiments could be reduced, for example by connecting two chemostat vessels to the same media reservoir, but subtle differences will still occur.

If washout were to be used in future to calculate μ_{max} for cultures containing these plasmids, more preliminary work would be required, and longer experimental times would be necessary. Alternatively, a different approach could provide the data necessary for calculating maximum specific growth rates. One such approach is the use of plate counts in order to determine μ_{max}. This is carried out by observing the length of time required to form macroscopically visible colonies, or by determining the radial increase at the edge of a colony (Salvesen & Vadstein, 2000). Batch culture can also be used for determining μ_{max} by measuring biomass and substrate concentrations and substituting data into the Monod equations (Tobajas & Garcia-Calvo, 2000). The benefits of either of these methods are reduced experimental times.

8.2 Future work that would further the main aim of the study

The sections above highlight the questions raised during the study, and possible ways of obtaining answers to them. The following sections cover the future of this area of research leading ultimately to the discovery of compounds that can cause destabilisation.

8.2.1 Is the level of instability observed for plasmids pOG04, pKO04, pOG4.003 and pKO4.003 enough to cause clearing of an infection?

Steady-state chemostat cultures of *E. coli* C2110 containing the plasmids pOG04, pKO04, pOG4.003 and pKO4.003 contained a high level of plasmid-free cells and formed unstable equilibria, indicating that there was a high plasmid loss rate. This may indicate that the plasmid-bearing cells were not being successfully maintained within the population. Repeating the steady-state experiments over a longer time period may cause a complete loss of the culture from the chemostat, purely as a result of plasmid instability. This may prove that plasmid destabilisation could work as an aid in restoring antibiotic sensitivity.

8.2.2 Can plasmid destabilisation be achieved by chemical means?

All the experiments carried out within this study involved the use of a genetic model of plasmid destabilisation. While this is beneficial in preventing additional variables caused by external factors, it cannot show whether chemicals are capable of plasmid destabilisation.

There are several chemicals that are known to affect DNA, although for obvious reasons they are highly toxic to humans as well as bacteria. For example, acridine orange is seen to bind to DNA, and in this capacity may be capable of disrupting plasmid inheritance (Lyles & Cameron, 2002), although it would most likely also affect chromosome replication, which would present difficulties in determining whether cell death was caused by plasmid destabilisation or general toxicity.

Other chemicals that could be studied include psoralen, which causes cross-linking of DNA, in turn affecting DNA replication (Bessho, 2003). Methylene blue causes single

strand breaks in RNA (Schneider *et al*., 1998), which may affect the partitioning system by disrupting protein production, although both of these chemicals will also affect the whole prokaryotic cell.

A chemical that may show a more specific activity is plumbagin - a natural compound extracted from plants of the *Plumbagineae* and *Droseracea* families (Chemical Selection Working Group, 1999). Its effects on antibiotic resistance plasmids have been noted in several publications (Lakshmi *et al*., 1987; Durga *et al*., 1990).

A study on a new antimicrobial compound brodimoprim suggested that its antimicrobial action was caused by the disruption of plasmid transfer between cells. *E. coli* cells treated with sub-minimal inhibitory concentrations of brodimoprim lost 9% of their high molecular weight low-copy number plasmids and 23% of their low molecular weight high-copy number plasmids. Bromidoprim was also seen to reduce conjugation by 50% when added to the culture at five times the minimal inhibitory concentration (Marchese *et al*., 1996).

8.2.3 A potential screen for a plasmid destabilising agent

The work carried out in this study has indicated that plasmid stability systems could be potential targets for antimicrobial agents. In order to capitalise on this potential, a fast screening process would need to be devised that would allow compounds to be tested for destabilising properties.

The important factors required for a screen of this type are ease of use, short experimental times and a clear indication of whether a compound has the activity required. The easiest way of accomplishing these aims in a microbiological assay is with the use of a single agar plate, and an example of the way in which this could work is described below.

The galactose metabolism operon of *E. coli* is essential for the utilisation of galactose within culture medium. The first reaction in the metabolism is the production of galactose-1-phosphate from galactose by the galactokinase enzyme, a highly toxic compound if not broken down quickly by the transferase enzyme. Galactose is a simple

sugar, and preferentially used as a carbon source over more complicated sugars such as glycerol.

A stable plasmid containing only the gene coding for the galactokinase enzyme is constructed, and transformed into a mutant strain of *E. coli* lacking the galactose operon entirely. When plated onto culture medium containing both galactose and glycerol, galactokinase will be produced in order to utilise the simple carbon source, however the build up of galactose-1-phosphate will kill the cell. However, if the plasmid is lost, the cell will use the glycerol as the sole carbon source and will be able to grow and form a visible colony (Busby & Dreyfus, 1983; Dreyfus *et al*., 1985).

Therefore, if a compound suspected of causing plasmid instability is added to the plate, the growth of any colonies will indicate that the plasmid has been lost from the cells, and therefore the compound may be capable of causing plasmid destabilisation.

Another screen for plasmid loss has been analysed in yeast strains, where cells expressing the green fluorescent protein from a plasmid can be easily identified using flow cytometric analysis (Hegemann *et al*., 1999). Another group introduced the mercury hypersensitivity gene *merA* onto a bacterial plasmid. When the plasmid was transferred into a host cell lacking this gene, and plated onto mercury-containing agars, the cells died. When the plasmid was lost however, the cells were free to grow again (Mori *et al*., 1991). Also, cells containing a plasmid coding for the ß-galactosidase gene will form a blue colony in the presence of X-gal, while plasmid-free colonies will be white (Meima *et al*., 1996).

Finally, it has been noted that plasmid-free and plasmid-bearing cells grow to form different sized colonies as a result of plasmid burden (Park & Ryu, 1992; Lynch *et al*., 2000). While not as simple to detect as a colour change or the absence or presence of growth, it requires little alteration or construction of plasmids or mutant host strains.

8.3 Conclusion

The aim of this study was to determine whether reducing the efficiency of plasmid inheritance would produce a measurable increase in the number of antibiotic-sensitive cells within a bacterial culture.

In order to fulfil this aim, a series of plasmids were used that provided different levels of stability at a genetic level. These plasmids were transformed into cultures and grown using a number of different culture techniques, all with the aim of measuring the effects of instability on growth in various ways.

The batch culture method was a fast and simple technique, and was used to calculate the rate of plasmid loss per generation for the plasmid-bearing cultures. This was a useful parameter to determine as it gave an indication of the percentage of plasmid-free cells produced after each round of cell division. The results showed that the different plasmids studied in this work had different levels of stability; some were very stably inherited such as pALA1029 and pKO1029, others were lost frequently from the host cell such as plasmids pOG4.003 and pKO4.003. This was positive confirmation that the plasmids showed a good range of stability levels and would be useful as a genetic model of instability. This work also showed that changing the antibiotic resistance that the plasmid conferred seemed to affect the stability, a phenomenon that is so far unexplained.

Steady-state chemostat culture experiments were carried out in order to increase the similarity of the growth conditions to that of a clinical infection. The chemostat has been previously used as a model of a human gut (Freeman *et al*., 2003), although not as a model of infection. It was considered to be appropriate as the chemostat shares many characteristics with a chronic infection, notably the constant concentration of biomass within the culture vessel, as would be the case for an untreatable bacterial infection. The results obtained from this work showed that cultures bearing unstable plasmids show a high percentage of antibiotic sensitivity, suggesting that plasmids are lost over the course of the experiment. This is a very compelling result, as it implies that decreasing the stability of antibiotic resistance plasmids will render a bacterial culture sensitive to antibiotics. This section of work also highlighted the intriguing notion that

plasmids containing no stability system were more unstable than plasmids containing a faulty stability system that caused plasmid dimers to enter daughter cells.

The final section of work was the determination of the maximum specific growth rate of plasmid-containing cultures by the use of chemostat washout. This was designed to be a very specific determination of the effect of plasmid instability on cell growth. Problems were encountered with this method, most importantly that washout experiments are not directly repeatable, leading to very few statistically significant results. However, the experiments did show that the growth rates of cultures containing unstable plasmids were disadvantaged when grown in the presence of antibiotics.

Many papers have been published on almost all aspects of plasmid stability, but so far no group seems to have considered how important this may be to the pharmaceutical industry. In the fight to win the war against antibiotic resistance, the only stone left unturned appears to be that of the bacterial plasmid, the structure in a bacterial cell frequently responsible for conferring antibiotic resistance. This study has shown that reducing the ability of plasmids to partition correctly to daughter cells during cell division can reduce the level of antibiotic resistance in a culture. While further work needs to be carried out in this area, it is a hopeful thought that the human race may have one last trick up its sleeve in order to prevent the return of a world without antibiotics.

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APPENDICES

A Abbreviations for plasmid diagrams given in Figs. 2.1 to 2.11 and Figs. 3.4 and 3.5

Abbreviation	Description
*Bam*HI	recognition site for the enzyme *Bam*HI
*Eco*RI	recognition site for the enzyme *Eco*RI
*Hin*cII	recognition site for the enzyme *Hin*cII
*Hin*dIII	recognition site for the enzyme *Hin*dIII
*Pst*I	recognition site for the enzyme *Pst*I
*Sal*I	recognition site for the enzyme *Sal*I
Ap^r	ampicillin resistance gene (ß-lactamase class A TEM-1 enzyme)
Ap'	partial, and therefore non-functional ampicillin resistance gene
Cm^r	chloramphenicol resistance gene (type I chloramphenicol acetyl transferase)
Tc^r	tetracycline resistance gene (TetC energy-dependent efflux)
Tc'	partial, and therefore non-functional tetracycline resistance gene
ori	replicon for replication of plasmid (from pMB1)
rop	reduces plasmid copy number (from pMB1)
*par*A	part of plasmid stability system (from bacteriophage P7)
*par*B	part of plasmid stability system (from bacteriophage P7)
*rep*A	RepA protein gene (from bacteriophage P7)
*ori*P7	origin recognised by RepA protein (from bacteriophage P7)
RK2 *ctl*	plasmid stability region (from plasmid RK2)
RK2 *ctl* O_B3^-	plasmid stability system lacking O_B3 site (from plasmid RK2)
IS*1*-L	left insertion sequence from Tn*9*
IS*1*-L'	partial, and therefore non-functional left insertion sequence from Tn*9*
IS*1*-R	right insertion sequence from Tn*9*
IS*1*-R'	partial, and therefore non-functional right insertion sequence from Tn*9*
IR-R	right inverted repeat of Tn*3*
*trp*E/*trp*D'	expressed *trp*E gene and a truncated *trp*D gene

B Abbreviations for bacterial strain descriptors given in Table 2.1, p. 28

Abbreviation	Description
polA1	A strain lacking alpha 1 DNA polymerase (*polA* gene)
$PolA^+$	A strain containing alpha 1 DNA polymerase (*polA* gene)
$PolA^-$	A strain lacking alpha 1 DNA polymerase (*polA* gene)
his	A mutation affecting histidine biosynthesis
rha	A mutation affecting rhamnose biosynthesis
$P2^s$	The presence of phage P2*s* within the genome
endA1	A mutation affecting endonuclease 1
hsdR17 $r_K^-m_K^-$	A mutation in the restriction/methylation pathway
supE44	Suppressor of amber (UAA) mutation
thi-1	A mutation affecting thiamine biosynthesis
recA1	A mutation affecting a general recombination gene
gyrA	A mutation affecting DNA gyrase
Nal^r	Nalidixic Acid resistance
relA1	A mutation that eliminates the stringent factor allowing relaxation of phenotype
Δ (*lacIZYA-argF*) *U169*	A delection removing the section of the genome containing the genes *lacI*, *lacZ*, *lacY*, *lacA* and through to *argF*.
deoR	Gene present that allows uptake of large plasmids
Φ80*dlac*Δ(*lacZ*)M15	Partial deletion of the *lacZ* gene allowing selection on X-Gal/IPTG plates.

C Abbreviations for general scientific terms used in the thesis

Abbreviation	Description
l	measurement of volume – litres
ml	measurement of volume – millilitres (10^{-3} litres)
µl	measurement of volume – microlitres (10^{-6} litres)
nl	measurement of volume – nanolitres (10^{-9} litres)
M	measurement of concentration – moles
mM	measurement of concentration – millimoles (10^{-3} moles)
µM	measurement of concentration – micromoles (10^{-6} moles)
nM	measurement of concentration – nanomoles (10^{-9} moles)
m	measurement of length – meters
mm	measurement of length – millimetres (10^{-3} metres)
µm	measurement of length – micrometres (10^{-6} metres)
nm	measurement of length – nanometres (10^{-9} metres)
g	measurement of weight – grams
mg	measurement of weight – milligrams (10^{-3} grams)
µg	measurement of weight – micrograms (10^{-6} grams)
ng	measurement of weight – nanograms (10^{-9} grams)
^{o}C	measurement of temperature – degrees Celcius
g	measurement of inertia – gravity
RPM	measurement of rotation – revolutions per minute
Ci	measurement of radioactivity - Curie
h	measurement of time - hours
OD_{600}	measurement of optical density at 600nm
NaCl	Sodium chloride
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
DNA	Deoxyribonucleic acid
$CaCl_2$	Calcium chloride
NaI	Sodium iodide
$MgCl_2$	Magnesium chloride